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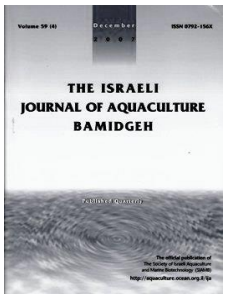

Antifungal Activity of *Magnolia officinalis* Derived Magnolol and Honokiol on Membrane Disruption of *Saprolegnia parasitica*

Yang Feng[1, #], Jing Duan[1,#], Cunbin Shi [2,*] , Xiaoli Huang [1,*], Kaiyu Wang [3], Yongqian Deng [4], Yi Geng [3], Ping Ouyang [3], Defang Chen [1], Shiyong Yang [1]

[1] *Department of Aquaculture, College of Animal Science & Technology, Sichuan Agricultural University, Wenjiang, 611130, Sichuan, China*
[2] *Key Laboratory of Fishery Drug Development, Ministry of Agriculture, Key Laboratory of Aquatic Animal Immune Technology, Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, 510380, Guangdong, China*
[3] *College of Animal Science and Veterinary Medicine, Sichuan Agricultural University, Wenjiang, 611130, Sichuan, China*
[4] *Department of Agriculture of Sichuan Province, Hongya, 620360, Sichuan, China*

Keywords: *Saprolegnia parasitica;* magnolol; honokiol; antifungal activity

Abstract

Saprolegniosis is an infectious disease caused by *Saprolegnia* sp. It may cause serious losses in fish farms and hatcheries in many countries. In previous research, *Magnolia officinalis* has been found to initiate the death of *Saprolegnia* sp. Magnolol and honokiol, two active substances in *M. officinalis*, may be responsible for the antifungal effects on *Saprolegnia* sp. The present study centered on the anti-fungal activity and mechanism of magnolol and honokiol on *Saprolegnia parasitica*. Results revealed that magnolol and honokiol displayed effective anti-fungal activity on *S. parasitica*, which caused their mass mortality. More intuitive research has shown that magnolol and honokiol have evolved mechanisms that can destroy the cell membranes of *S. parasitica*, and cause exosmosis of their cytoplasmic saccharides and proteins, and cause mortality. Magnolol and honokiol exhibited minimum inhibitory concentration values of 976.6 ug/mL and 587.4 ug/mL concentrations, respectively, and the minimum inhibitory time of both was 2 hours. This study provides a clearer understanding of the function of *M. officinalis* against *Saprolegnia* sp.

* Corresponding authors: C.B.Shi; Email: shicunbin2006@163.com ;
X.L.Huang; email: hxldyq@126.com
#The first two authors contributed to the work equally.

Introduction

Saprolegniosis is a major disease in farmed and wild fish in many countries. As it is an infectious disease caused by *Saprolegnia* sp, saprolegniosis may cause serious losses in fish farms and hatcheries (Eli et al. 2011). The hyphae of *Saprolegnia* sp. usually grow through the epidermis and dermis into the musculature, and then cause dermatitis and myositis (Hussein et al. 2002). Severe saprolegniosis can cause fish mortality (Roberts 2010). Therefore, detection and treatment of saprolegniosis is a top priority. Malachite green was the most effective therapeutic agent in early years to control and prevent *Saprolegnia* zoospore production and cyst germination. However, due to its carcinogenic and toxicological effects, the use of malachite green has been prohibited in commodity fish (Alderman 1985). Although sodium chloride, formalin, hydrogen peroxide, and ozone are currently used in aquaculture, the huge volume of chemicals required, and the resulting environmental pollution, make it impractical for their use in large ponds (Schreier et al. 1996). The abuse of these drugs has resulted in the exacerbated environmental pollution and resistance of *Saprolegnia* sp. (Rowland et al. 2006). In Eastern and Southeast Asia, traditional Chinese herbal medicines have been used to prevent diseases for thousands of years (Chanu et al. 2012). Currently, the new role of ancient Chinese medicine has been discovered, and many natural plant compounds have shown to be effective in fighting fish diseases (Wang 2010). Our previous study found that *Magnolia officinalis* has an inhibitory effect on *Saprolegnia* sp. (Huang et al. 2015), but the inhibitory effect mechanism is still unknown. Magnolol and honokiol, active substances obtained from *M. officinalis*, have been found to have a good bactericidal and anti-fungal effects (Bae et al. 1990, Jang et al. 1993; Bang et al. 2000; Kyeongsoo et al. 2000; Greenberg et al. 2007; Yang et al. 2017). However, whether magnolol and honokiol have an inhibitory effect on *Saprolegnia* sp. and how they function is unclear.

The research questions of the present study were centered on the anti-fungal activity of two active substances of *M. officinalis*, magnolol and honokiol, to *S. parasitica*. Beyond that, the anti-fungal mechanism of these two active substances was further investigated.

Materials and Methods

Material preparation

Samples of *Saprolegnia* sp. were taken from hybrid sturgeon affected by saprolegniosis. The *internal transcribed spacer 1* (*ITS 1*) was amplified and sequenced (940 bp). The sequence was submitted to NCBI (https://www.ncbi.nlm.nih.gov/) and obtained the accession numbers MG597031. Homology analysis showed that the *Saprolegnia* sp. was clustered to *S. parasitica*. Before the experiment, *S. parasitica* was cultured onto potato-dextrose agar (PDA) medium and incubated at 20^0C for use.

Magnolol (purity = 98%) and honokiol (purity = 98%) were purchased from Xi'an Huilin Bio-Tech. Co., Ltd and were both almost insoluble in aqueous solution (HongyiZhang et al. 1997). To avoid unnecessary interference in the experiment, 50% ethanol was used to dissolve magnolol and honokiol, which was nontoxic to *S. parasitica* (Table 1). The remaining chemicals were of analytical grade and obtained from local companies.

Table 1. The toxic effects of various solvents on the growth of *S. parasitica*

Solvent	*Suppress ability*	*Solvent*	*Suppress ability*
Distilled water	+	*50% Ethanol*	+
100% Ethanol	-	*Ethyl acetate*	-
Acetone	-	*Positive control*	+
Ethyl acetate	-	*Negative control*	-
Petroleum ether	+		

Note: The medium without solvent as positive control, and without *S. parasitica* as negative control. '**+**' *S. parasitica* grew on the media; '**-**' no *S. parasitica* growth.

Experimental treatments

The experiment was divided into three groups: magnolol group (2.5×10^4 μg/mL magnolol), honokiol group (2.5×10^4 μg/mL honokiol), and control group (50% ethanol + PBS). The tests of each group were repeated 3 times. *S. parasitica* was flushed from the PDA medium with PBS for use. Then, 0.5 g *S. parasitica* and 20 mL treating fluid of each group were combined in a shaking incubator at 20^0C (Fig. 1). The treated *S. parasitica*

was collected for transmission electron microscopy (TEM) and Hoechst 33342/PI staining test, and the solution was used for the evaluation of concentration of extracellular soluble saccharide and protein.

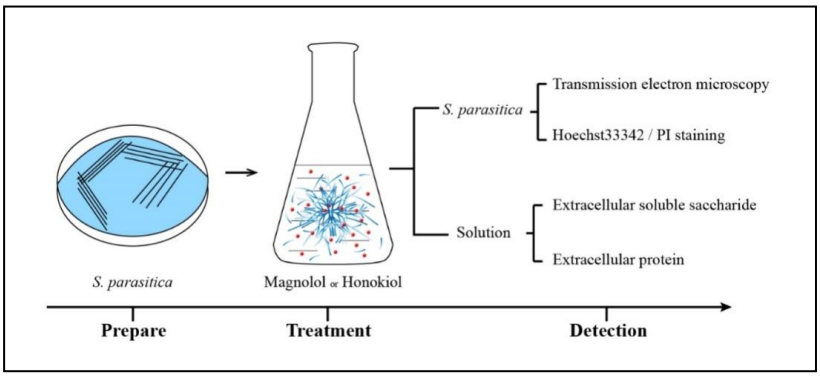

Fig. 1. Experimental design.

Hoechst33342 / PI staining

After 8 hours of treatment, fluid of *S. parasitica* was centrifuged at 8000 r/min and 0.1g and the precipitate washed with PBS three times. All procedures were performed based on the Hoechst 33342/PI Double Stain Kit manufacturer's protocol (Yeason, Shanghai, China). 0.4 mL cell staining buffer was added to resuspend the *S. parasitica*. 5 μL Hoechst 33342 stain and 5 μL PI stain was added then, and *S. parasitica* was incubated in an ice bath for 25 min. The experiment was conducted in the dark. Finally, *S. parasitica* was washed with PBS and observed using a Nikon Eclipse Ti-S fluorescence microscope system (Nikon, Tokyo, Japan). Blue fluorescence was observed at a wavelength of 450 nm, while red fluorescence was 630 nm. Based on the kit manufacturer's protocol, different fluorescence intensities represent different types of death: apoptosis = weak red fluorescence + strong blue fluorescence; necrosis = strong red fluorescence + weak blue fluorescence.

TEM observation

After 8 hours treatment, fluid of *S. parasitica* was centrifuged at 8000 r/min and 0.1g precipitate was fixed with 4% glutaraldehyde (4^0C) 3 to 6 h. After dehydration in graded alcohol, the tissues were embedded in Araldite. The blocks were sectioned in a microtome with a glass knife. Sections, 6575 nm thick, were placed in uncoated copper grids. The sections were stained with uranyl acetate, and post-stained with 0.2% lead citrate. The subcellular structure of *S. parasitica* was examined with Hitachi Hu-12A TEM (HITACHI, Tokyo, Japan)).

Determination of extracellular soluble saccharide

The extracellular soluble saccharide content was determined using the anthrone colorimetric method (Halhoul et al. 1972). During preparation of the experiment, the standard glucose solution was used to make a standard curve. After 0, 2, 7 hours' treatment, treating fluid of *S. parasitica* in each trial was centrifuged at 8000 r/min and 1 mL supernatant was drawn from the fluid. Then, 5 ml 0.2% anthrone reagent was added to the supernatant. Finally, the reaction mixture was measured in Thermo Scientific Microplate Reader Varioskan LUX (Thermo, Massachusetts, USA) at a wavelength of 630 nm, and the content of soluble saccharide was calculated according to the standard curve.

Determination of extracellular protein

Soluble protein content was detected by modified Coomassie brilliant blue G-250 staining method (Blakesley et al. 1977). During the experiment preparation, the bovine serum albumin standard solution was used to make a standard curve. After 0, 2, 7 hours treatment, treating fluid of *S. parasitica* in each trial was centrifuged at 8000 r/min and 1 mL supernatant was drew from the fluid. Then, 5 mL Coomassie brilliant blue G-250 was added in the supernatant. The reaction mixture was measured in Thermo Scientific Microplate Reader Varioskan LUX (Thermo, USA) at a wavelength of 595 nm, and the content of soluble protein was calculated according to the standard curve.

Inhibitory efficiency evaluation

Minimum inhibitory concentration (MIC) evaluation: The MIC of magnolol and honokiol on *S. parasitica* was determined using a 96-well microdilution. *S. parasitica* was cultured for amplification for 1 week in PDA at 20^0C. A Vortex mixer was used to agitate the subculture for 10 min to release the spores. The solution was filtered with three layers of sterile gauze. The final spore concentration was calculated using a hemocytometer adjusted to 1.0×10^4 CFU/mL with sterile water. The compound solutions were all prepared in serial two-fold dilutions in potato-dextrose broth within a dilution range from 1/2 to 1/1024. Each well contained 50μL grades of diluted compounds and 50 μL spore suspension of *S. parasitica*. In addition, one well acted as the positive control containing only 50 μL medium; another well contained only 50 μL magnolol or honokiol solution and acted as the negative control. The plates were incubated at 20°C for 48 h. Each compounds solution was tested in triplicate.

Minimum inhibitory time evaluation: *S. parasitica* was injected into magnolol solution (2.5×10^4 μg/mL magnolol) and honokiol solution (2.5×10^4 μg/mL honokiol) as described above. The treatment solution was placed in a shaking incubator at 20^0C. After 0, 2, 4 hours treatment, treating fluid of *S. parasitica* was centrifuged at 8000 r/min and 0.1g precipitate was washed with PBS. *S. parasitica* was resuspended and stained with Hoechst33342 / PI stain. Finally, *S. parasitica* was observed using a Nikon Eclipse Ti-S fluorescence microscope system (Nikon, Japan), and the fluorescence intensity was detected in Image Processing and Analysis in Java 1.6.0 (National Institutes of Health, USA).

Statistical Analysis

The results are expressed as the mean value and standard deviation. The significance of differences was analyzed by variance analysis. The analysis was performed using one-way analysis of variance while the *t*-test was applied to determine whether the differences between groups were significant (SPSS v.20.0, IBM Corp., Armonk, New York, USA). A value of $P < 0.05$ was considered significant, while a $P < 0.01$ was considered highly significant.

Results

Magnolol and honokiol caused death of S. parasitica

Compounds treatment caused an intense fluorescence of *S. parasitica* (Fig. 2). The fluorescence intensity of each treatment was higher than that of the control group. After merging blue and red fluorescence, it was found that two compounds induced scarlet fluorescence. Based on the kit manufacturer's protocol, magnolol or honokiol caused obvious necrosis in *S. parasitica*. Therefore, the results indicated that magnolol and honokiol had an effective lethal effect on *S. parasitica.*

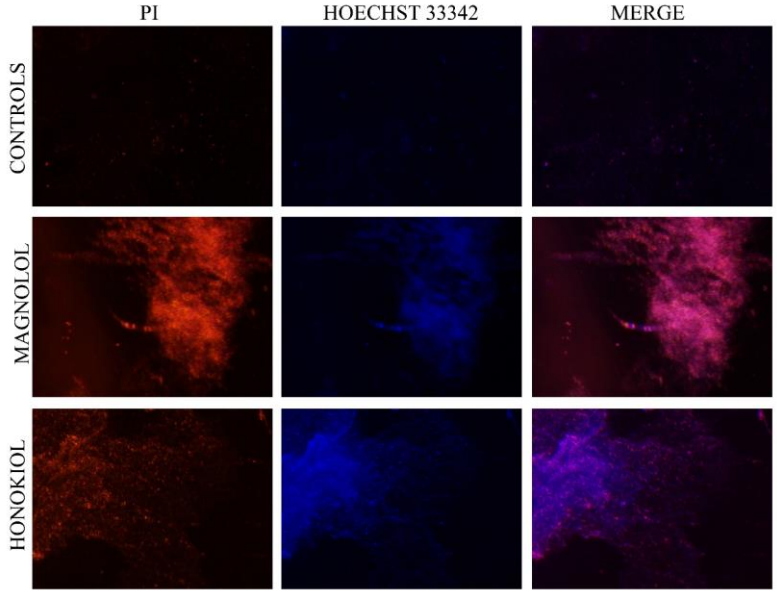

Fig.2 Hoechst33342 / PI staining of *S. parasitica* in different treatment
Red shows that *S. parasitica* has been stained with PI, and blue shows that *S. parasitica* has been stained with Hoechst 33342, Merge shows the two colours flattened.

Magnolol and honokiol damage the biomembrane of S. parasitica

Damage of *S. parasitica* was further confirmed by TEM. Magnolol and honokiol caused degeneration of organelles in *S. parasitica* under observation (Fig. 3). In the control group, the endoplasmic reticulum was arranged neatly, accompanied with the small vacuoles and liposomes in the cytoplasm (Fig. 3 A, B). Compared with the control group, some cell membrane of *S. parasitica* appeared to be affected by magnolol and honokiol and became obscured or disappeared (Fig. 3 C-F). In addition, mitochondrial membranes and vacuole membranes were also affected in varying degrees (Fig. 3 C-E). These results indicated that two compounds may rupture the membrane structure of *S. parasitica* and eventually induce cell death. Furthermore, the organelles of *S. parasitica* gradually disappeared and the cytoderm became thinner after magnolol and honokiol treatment. Secondary lysosomes appeared gradually in the cytoplasm after treatment with the compounds (Fig. 3 E).

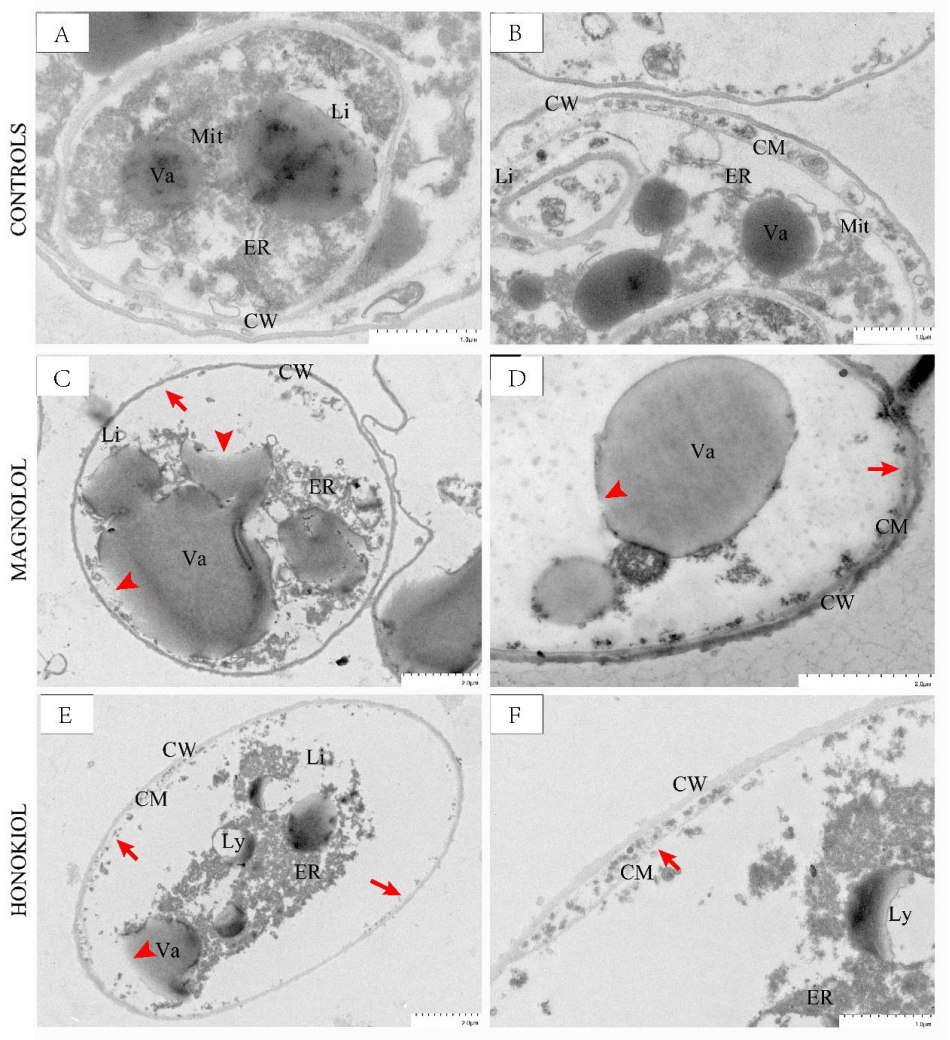

Fig. 3. Morphology of *S. parasitica* spores under TEM

A and B showed the morphology of *S. parasitica* of control group. Organelles arranged neatly in A, and the cell membrane intact and clear in B. C and D declare the situation of magnolol group, the organelles become cluttered, and mitochondria disappeared. C appeared bubble fusion in the cytoplasm. D showed the cell membrane damage site. E and F declare the situation of honokiol group, E showed secondary lysosomes emerged in the cytoplasm, and mitochondria disappeared, and F was a partial enlargement of E and we also found that the cell membrane was blurred and damaged. Arrow: cell membrane obscured or disappeared; arrowhead: vacuole membranes obscured or disappeared CW: cell wall; CM: cell membrane; ER: endoplasmic reticulum; Va: vacuole; Li: liposome; Ly: lysosome; Mi: Mitochondria. n=3 for each group.

The cytoplasmic contents released outside of S. parasitica

The results above show that if the cell membrane has been damaged and causes the death of *S. parasitica*, so the cytoplasm and the cytoplasmic content may infiltrate into the extracellular during this process. Therefore, changes of cytoplasmic content, soluble saccharide and protein, in the solution have been detected. Results showed that mass soluble saccharide appeared in the solution after magnolol or honokiol treatment (Fig. 4), and displayed significant differences compared to the control. In addition, the soluble protein in the solution was enhanced and was significantly different from the control group (Fig. 5). All the results here have indicated that the two compounds, magnolol or honokiol may damage the cell membrane of *S. parasitica*.

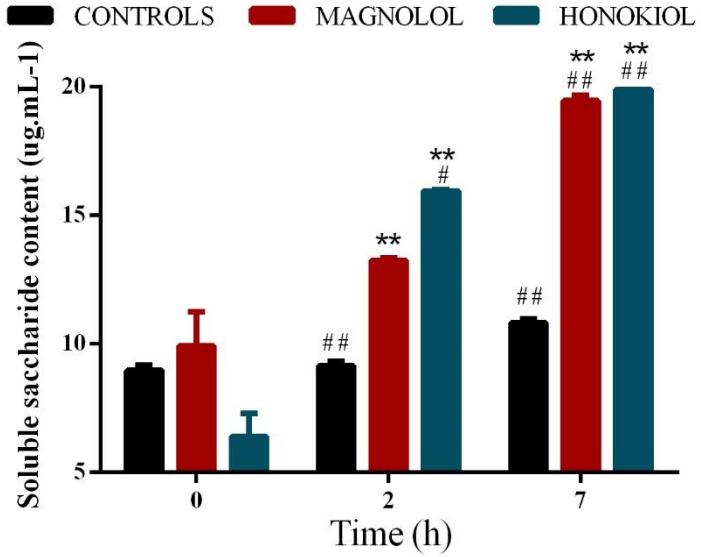

Fig. 4 Changes of saccharide in different treatment
* or ** represents a significant difference (P<0.05) or a highly significant difference (P<0.01) between the control and compounds group. # or ## represents a significant difference (P<0.05) or a highly significant difference (P<0.01) between different treatment times in the same group, n=3 for each group.

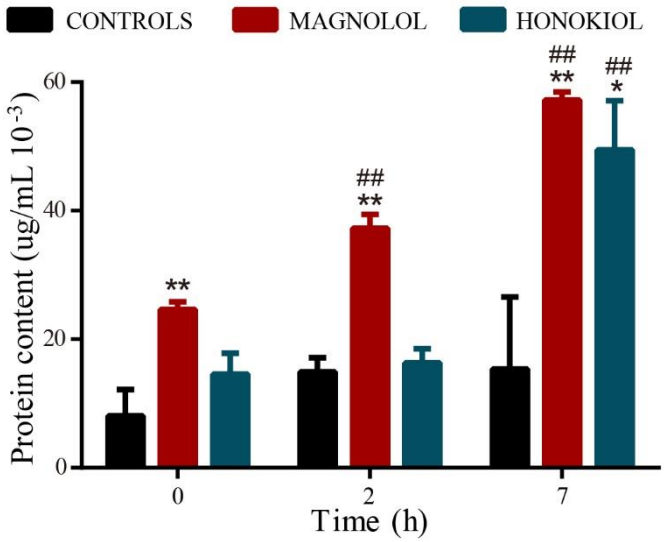

Fig. 5. Changes of protein in different treatment
* or ** represents a significant difference (P<0.05) or a highly significant difference (P<0.01) between the control and compounds group. # or ## represents a significant difference (P<0.05) or a highly significant difference (P<0.01) between different treatment times in the same group, n=3 for each group.

Inhibitory efficiency evaluation of magnolol and honokiol on S. parasitica

The efficiency of the compounds was evaluated to see if they could be introduced for the treatment of saprolegniosis. The MIC of magnolol and honokiol was determined. Results showed that the MIC of magnolol and honokiol exhibited values of 976.6 ug/mL and 587.4 μg/mL concentrations, respectively (Fig. 6). The fluorescence intensity of hyphae showed that both magnolol and honokiol caused significant deaths of *S. parasitica* after 2 hours (Fig. 7).

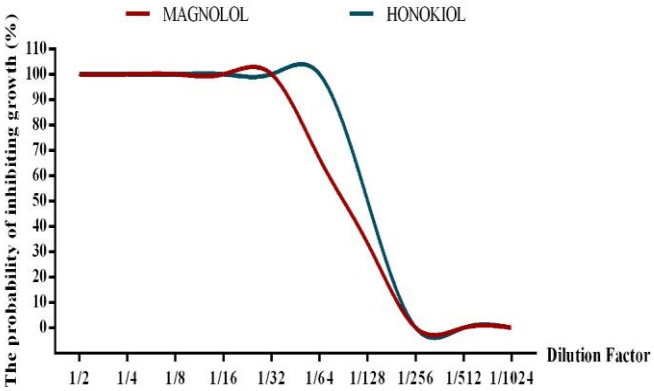

Fig.6. Antifungal activity of different concentrations of magnolol and honokiol on *S. parasitica*
The X axis represents the dilution factor of compounds, ie., drug concentration: 1/2=2.5×10^4 ug/mL, 1/1024=49 ug/mL

Drug	Species	MIC (ug mL^{-1})
Magnolol	*S. parasitica*	976.6
Honokiol		587.4

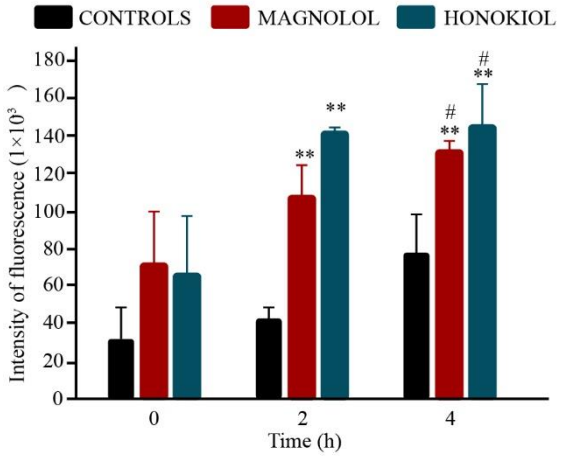

Fig. 7. The efficacy of different time of magnolol and honokiol in *S. parasitica*
** represents a highly significant difference (P<0.01) between the control and compounds group. # represents a significant difference (P<0.05) between 0 h and others, n = 3 for each group.

Discussion

There are thousands of species of Chinese herbs with a multitude of functions. A previous study also revealed that *M. officinalis* has a good inhibitory effect on *Saprolegnia* sp. (Huang et al. 2015) and may be an alternative to malachite green (Alderman 1985), formalin (Akpoilih et al. 2010), or other drugs. To further explore the role of *M. officinalis* on *Saprolegnia* sp., the effect of two active ingredients extracted from *M. officinalis* on *S. parasitica* was studied here. The results showed that both magnolol and honokiol are highly lethal to *S. parasitica*.

Many studies showed that magnolol and honokiol could induce bacterial or fungal death through multiple pathways and could suppress the bacteria or fungi by inhibiting cell division (Liu et al. 2014), gene transcription (Chen et al. 2006, Tse et al. 2007, Chuang et al. 2011), protein translation (Son et al. 2000, Matsuda et al. 2001), or generation of cellular components (Sun et al. 2015). Our experiment documented that magnolol and honokiol kills *S. parasitica* directly, rather than inhibiting cell proliferation. Furthermore, the results also showed that cell membranes of *S. parasitica* became obscure, and some even ruptured and disappeared. Other studies have demonstrated that magnolol and honokiol change the cellular membrane permeability and lead to necrosis (Greenberg et al. 2007, Joo et al. 2014, Yang et al. 2017), which is similar to our observations. On the other hand, macromolecules cannot penetrate the cell membrane under normal circumstances (Skou 1965, Jain et al. 1969). The outflow of

saccharide and protein in this paper also indicated destruction of the *S. parasitica* cell membrane during compounds treatment (Fig. 8).

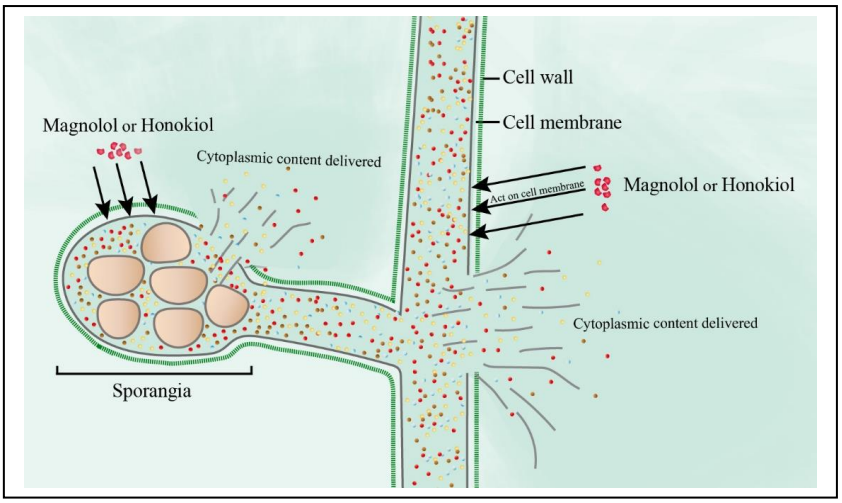

Fig. 8. The mechanism of magnolol and honokiol induced cell death in *S. parasitica*

In fish suffering from saprolegniosis a large amount of *Saprolegnia* sp. was generally attached on the skin surface. Therefore, in aquaculture cure of saprolegniosis is problematic. In this paper, MIC and the minimum effect time for magnolol and honokiol were tested for their suitability in aquaculture. Although MIC was higher than that of drugs such as copper sulphate (Sun et al. 2014), its application in aquaculture still is very promising due to the availability of the herb magnolia.

Conclusions

The focus of our research in this study was on the anti-fungal effect of two active substances of *M. officinalis*, magnolol and honokiol to *S. parasitica*. From this study, we conclude that magnolol and honokiol showed effective antifungal activity against *S. parasitica.* This study shows theoretical evidence that *M. officinalis* could be a potential drug for the treatment of saprolegniosis.

Acknowledgements

We thank all authors for their contribution to this article. Y F and J D cultivated *S. parasitica* and completed the experiment. Y F participated in the data analysis. XL H and CB S participated in the design of the experiment and drafting the manuscript. KY W, YQ D, Y G, P OY, DF C, and SY Y coordinate research and participate in revision of manuscript. All authors read and approved the final manuscript. This research was supported by Open fund project of Key Laboratory of Fishery Drug Development (Grant number 201502).

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