Dihydroartemisinin Extract from *Artemisia hedinii* inhibits spore germination of *Alternaria alternata* involving membrane peroxidation

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Research Article

Keywords: *Artemisia hedinii*, Dihydroartemisinin, Membrane peroxidation, *Alternaria alternata*, Spores

Posted Date: February 18th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1355274/v1>

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Abstract

Dihydroartemisinin (DHA) extract is expected to be useful as a biological pesticide for fungal disease control. This study preliminarily explored the mechanism for inhibiting spore germination of *Alternaria alternata* by DHA extract. The results showed that the EC₅₀, EC₉₀, MIC and MFC of DHA extract on the spores of *A. alternata* were 1.63 mg/mL, 3.14 mg/mL, 4 mg/mL and 8mg/mL, respectively. The cell membrane permeability of spores treated with DHA extract was enhanced and the integrity was decreased. Meanwhile, the contents of reactive oxygen species (ROS) and malondialdehyde (MDA) increased significantly, indicating that membrane peroxidation occurred in the spores. Significant changes in catalase (CAT) activity were observed, which also demonstrated that an oxidation reaction took place within the spores. These results indicated that the DHA extract produced ROS in the spores, which caused membrane peroxidation and destroyed the membrane, thus, inhibited spores germination of *A. alternata*. The antifungal activity against *A. alternata* mechanism of DHA could pave the way for the development of control of tobacco brown spot and other fungal diseases.

Introduction

Tobacco Brown Spot Disease caused by *Alternaria alternata* is one of the main causes of tobacco production reduction. Previous studies in our laboratory found that the extract of *Artemisia hedinii* had antifungal activity against *A. alternata*, and its main antifungal component was dihydroartemisinin (DHA) (Li 2013, XU et al. 2018). *Artemisia hedinii* is an annual herb, which has a strong odor. And it is a traditional Chinese medicine, which contains flavonoids, terpenoids, coumarins and other components, and has significant insecticidal and antifungal activities (Bora et al. 2011).

DHA, an important artemisinin derivative, is widely used as an intermediate of other artemisinin derivatives. It is also an excellent antimalarial drug. Its potency is 10 times that of artemisinin, and it is more stable than artemisinin (Arinaitwe et al. 2009, Wooa et al. 2010, Tu 2011). The antimalarial mechanism of DHA is still unclear. It is now generally believed that the antimalarial effect of DHA is related to its peroxide bridge structure (Zhang et al. 2008). The free radicals formed when the peroxide bridge breaks cause the plasmodium protein to alkylate and die. In our previous studies, it was found that DHA could destroy the cell membrane of the mycelia and spores of *A. alternata* and reduce the content of ergosterol in the mycelia. It was also found that DHA promotes spore production of *A. alternata*, but inhibited spore germination (XU et al. 2018). At present, there is no report on the antifungal mechanism of DHA. The purpose of this study was to explore the antifungal mechanism of DHA and provide theoretical support for the application of DHA in the prevention and control of fungal diseases. This study aims to investigate whether the mechanism for inhibiting spore germination of *A. alternata* by DHA extract is due to membrane lipid peroxidation within *A. alternata* spores by detecting intracellular reactive oxygen species (ROS), intracellular and extracellular malondialdehyde (MDA) content and catalase (CAT) activity.

Materials And Methods

Fungal species and materials

A. alternata was provided by Hubei institute of tobacco science and preserved on potato dextrose agar (PDA) at 28 ± 2 °C. The concentration of spores was adjusted to 1×10^7 spores/mL by hemocytometer. *A. hedinii* (Bozhou, Anhui) was provided by Hubei institute of tobacco science.

Smashing tissue extractor (ZHBE-50T) was produced by Henan Zhijing biotechnology co., LTD (Henan, China). Thermostatic magnetic stirrer (DF-101S) was produced by Wuhan Keer Instrument Equipment Co., Ltd. Optical microscope (BX43) was produced by Olympus corporation (Tokyo, Japan).

Chemicals and kits

DHA (98%), guaiacol (98%) and 2-thiobarbituric acid (98%) were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Anhydrous ethanol was obtained from Zhengzhou Paini Chemical Reagent Factory (Zhengzhou, China). Tween-80 was obtained from Tianjin Baishi Chemical Co., Ltd (Tianjin, China). Propidium iodide (95%) was purchased from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Hydrogen peroxide (H₂O₂, 30%) was purchased from Xilong Chemical Co., Ltd (Guangdong, China). Dipotassium hydrogen phosphate and monopotassium phosphate were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The active oxygen detection kit was purchased from Beyotime Biotechnology Research Institute (Shanghai, China). PDB medium was produced from Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co., Ltd.

Sample Preparation

The DHA extract suspension was prepared according to the following operating procedures. At first, DHA was extracted from dried *A. hedinii* powder by homogenate extraction (You et al. 2021). *A. hedinii* powder was mixed with anhydrous ethanol at a solid-liquid ratio of 1:22 g/mL. After mixing, 60 s was extracted with a smashing tissue extractor. The solution then was soaked for 34 min, filtered and concentrated to obtain the extract solution. Next, add 7.5 mL Tween 80 into 50mL water, stir to dissolve, and then slowly add 20 mL extract solution while stirring. After stirring for 5 min, magnetic stirring was performed at 90°C at a constant temperature. After 30 min, the heating was stopped. After stirring for 60 min, the solution was supplemented with water to 50 mL to obtain the DHA extract suspension.

The DHA standard suspension was prepared using DHA standard instead of the extract to perform a positive control test.

The DHA concentrations in the DHA extract suspension and the DHA standard suspension were 15 mg/mL.

Antifungal activity detection

The antifungal activity of DHA extract against *A. alternata* spores was detected by spore germination test. 100 µL spore suspension and 100 µL PDB medium were mixed, and DHA extract suspension and water were added to the mixture until the total volume was 400 µL, and the DHA concentrations were 0.25, 0.5, 1, 2 and 3 mg/mL. Add 50 µL of the mixture to a clean concave glass slide, and the concave glass slides were placed in 15 cm diameter Petri dishes, moisturized and cultured at 28°C for 6 h. Spore germination was observed under a microscope, and three visual fields were randomly selected for each concave glass slide. Spore whose length of the germ tube exceeds 1/2 of the short axis is considered germinated. The same concentration of DHA suspension was used as positive control. The experimental group without DHA extract suspension was used as blank control. The formula of spore germination rate and inhibition rate of spore germination is as follows:

$$\text{spore germination rate} = (A/B) \times 100\%$$

$$\text{Inhibition rate of spore germination} = (C - D)/C \times 100\%$$

A— Number of germination spores

B— The total number of spores observed

C— Spore germination rate in blank control group

D— Spore germination rate of treatment group

Determination of MIC and MFC

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of DHA extract suspension and DHA suspension against *A. alternata* spores were determined(Chen et al. 2019). 0.5 mL spore suspension and 0.5 mL PDB medium were mixed with some DHA extract suspension and water. The total volume of the mixture was 3 mL, and the DHA concentrations were 1, 2, 4, and 8mg/mL. After the mixture was incubated at 28°C for 6 h, the spore germination was observed under a microscope. The minimum DHA concentration of the experimental group without spore germination was the MIC. All spores in the experimental group without spore germination were washed with water for three times and inoculated on PDB medium, cultured at 28°C for three days. The minimum DHA concentration of the experimental group without mycelium growth was MFC. DHA suspension was used to replace DHA extract suspension for the same operation to detect the MIC and MFC of DHA suspension.

Release of cellular constituents

The release of cellular constituents was assessed by measuring the nucleic acid content of the extracellular solution using ultraviolet Spectrophotometer (Zhang et al. 2017). Spores were washed three times in PBS (0.1 mol/L, pH=7) and then resuspended with PBS. A certain amount of DHA extract suspension was mixed with 5 mL PDB and 5 mL spore suspension, and then PBS was used to volume the mixture to 20mL, the concentration of DHA in the mixture were 0, 1/2 MIC, MIC and MFC. When the mixture was shake cultured at 28°C and 120 rpm for 0, 0.5, 1 and 2 h, 5 mL samples were taken, and take the supernatant after centrifugation at 10,000 rpm for 10 min. The absorbance of supernatant was measured at 260 nm by ultraviolet spectrophotometer with PBS as blank control. DHA suspension was used to replace DHA extract suspension for the same operation to detect the effect of DHA suspension on the release of cellular constituents.

Assays for cell membrane integrity

The cell membrane integrity of the *A. alternata* spores treated with the DHA extract suspension was determined by the fluorescence value of propidium iodide (PI) staining(Zhu et al. 2019). The method of treating *A. alternata* spores with the DHA extract suspension is the same as Release of cellular constituents. The treated spores were collected by centrifugation (4°C, 10000 rpm, 10 min), washed three times with PBS (50 mmol/L, pH=7), and re-suspended in 1 mL PBS. 10 µL 1 mg/mL PI reagent was added into 1 mL spore suspension, stain in water bath at 37°C for 5 min, then wash with PBS for 3 times to remove the excess stain, and finally resuspend the spores in 5 mL PBS to prepare the stained spore suspension. The fluorescence value of the stained spore suspension was measured by fluorescent photometer (excitation wavelength was 546 nm, emission wavelength was 550 nm). DHA suspension was used to replace DHA extract suspension for the same operation to detect the effect of DHA suspension on the cell membrane integrity.

Assays for ROS

Reactive oxygen species (ROS) content in spores was detected by ROS detection kit(Tao et al. 2018). The method of treating *A. alternata* spores with the DHA extract suspension is the same as Release of cellular constituents. The treated spores were collected by centrifugation (4°C, 10000 rpm, 10 min), washed three times with PBS (50 mmol/L, pH=7). After washing, 5 mL DCFH-DA diluent (10 µM /L) was added to the spores to make the concentration of spores 10^6 - 10^7 Spores/mL, and the spores were shaken well and cultured at 28°C for 0.5 h. The spores were collected by centrifugation (4°C, 10000 RPM, 10 min), then washed with PBS for 3 times to remove the DCFH-DA that did not enter the cells, and resuspend the spores with 5 mL PBS. The fluorescence value of the spore suspension was measured by fluorescent photometer (excitation wavelength was 488 nm, emission wavelength was 525 nm). DHA suspension was used to replace DHA extract suspension for the same operation to detect the effect of DHA suspension on the ROS content.

Determination of MDA contents

The content of intracellular and extracellular MDA was determined by 2- thiobarbituric acid (TBA) method(Tao et al. 2014, Zhou et al. 2014). The method of treating *A. alternata* spores with the DHA extract suspension and DHA suspension are the same as Release of cellular constituents. The treated spore mixture was centrifuged (4°C, 10000 rpm, 10 min), and the supernatant was used to measure the extracellular MDA content, and the spores were collected and broken to detect the intracellular MDA content. The collected spores were washed with PBS (50 mmol/L, pH=7.2) for three times, and then ground in ice bath for 0.5 h to make them broken. Then 10 mL PBS was added for centrifugation, and the supernatant was collected to detect the intracellular MDA content. 2 mL supernatant was mixed with 5 mL 10% trichloroacetic acid (containing 0.6% 2-thiobarbituric acid), reacted in boiling water for 15 min, then cooled in ice bath to stop the reaction. After centrifugation, the absorbance of supernatant was measured at 450, 532 and 600 nm. The MDA content was calculated as follows:

$$C = (6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}) \times V_1 \div (W \times V_2 \div V_3)$$

C—MDA content, $\mu\text{M/g}$;

V_1 —The total volume of the reaction system, mL;

V_2 —Liquid volume of supernatant used for detection, mL;

V_3 —Total volume of supernatant, mL;

W—Sample weight, g.

CAT activity test

The method of treating *A. alternata* spores with the DHA extract suspension and DHA suspension was the same as Release of cellular constituents. The treated spores were washed three times with PBS (50 mmol/L, pH=7.2) at 4°C. After the spores were ground in ice bath, 10 mL PBS was added for centrifugation, and supernatants were collected as crude enzyme solution.

The reaction system for CAT activity detection was as follows: 0.3% hydrogen peroxide solution 1 mL, PBS (50 mmol/L, pH=7.2) 1.5 mL, crude enzyme solution 0.5 mL(Tao et al. 2018). In blank control group, pure water was used instead of crude enzyme solution. After 15 s of reaction, the absorbance of the reaction system was measured at 240 nm as the initial value, and then the absorbance was measured every 30 s for 10 consecutive times. The CAT activity was calculated as follows:

$$\text{CAT activity (U.g}^{-1}\text{prot)} = \Delta\text{OD}_{240} \times V / (0.1\Delta t \times V_s \times W)$$

ΔOD_{240} — Change in absorbance;

Δt — Reaction time;

V_s — Volume of crude enzyme solution for detection;

W — Sample weight.

Statistical analysis

All data were obtained through three independent replicates and expressed as mean $\pm$ SD (standard deviation). Origin2018 software was used for drawing and SPSS statistical software was used for data analysis.

Results

Antifungal activity

As shown in Table 1, there was no significant difference in the inhibition rate of spore germination between the two drugs when DHA concentration was below 2 mg/mL, and the inhibition rate of spore germination of DHA standard was significantly higher than that of DHA extract when DHA concentration reached 2 mg/mL. According to the data shown in Table 1, the regression equation of toxicity of DHA extract to *A. alternata* spores was $Y_1 = 26.487X + 6.711$, $R^2 = 0.991$, the EC_{50} and EC_{90} of DHA extract against *A. alternata* spores were 1.63 mg/mL and 3.14 mg/mL, respectively. The regression equation of toxicity of DHA standard on *A. alternata* spores was $Y_2 = 31.524X + 3.563$, $R^2 = 0.976$, the EC_{50} and EC_{90} of DHA standard against *A. alternata* spores were 1.47 mg/mL and 2.74 mg/mL. The toxicity of DHA standard to the spores of *A. alternata* was stronger than that of DHA extract.

Table 1 Antifungal activity of DHA extract and DHA standard

DHA concentration (mg/mL)	Inhibition rate of spore germination (%) [*]	
	DHA extract	DHA standard
0.25	10.84±3.19 ^f	9.49±1.36 ^f
0.50	19.04±3.76 ^e	18.45±6.38 ^e
1.00	36.23±5.15 ^d	34.86±4.04 ^d
2.00	62.76±5.18 ^c	76.78±4.53 ^b
3.00	83.47±3.37 ^b	92.52±2.02 ^a

^{*} Values are presented as mean ± SD.

^{a-e} Significant differences at $P < 0.05$ level.

Determination of MIC and MFC

As shown in Table 2, in the experimental group of DHA extract, spore germination was completely inhibited when the DHA concentration reached 4 mg/mL, and the MIC was 4 mg/mL. The mycelial growth was completely inhibited when the DHA concentration reached 8 mg/mL, indicating that spores were completely killed at this concentration, and the MFC was 8 mg/mL. In the same way, the MIC and MFC of DHA standard are both 4 mg/mL.

Table 2 The MIC and MFC of DHA extract and DHA standard

project	DHA concentration [mg/mL]	Spore germination	Mycelial growth
DHA extract	2	+	
	4	-	+
	8	-	-
	16	-	-
DHA standard	1	+	
	2	+	
	4	-	-
	8	-	-

^{*} spore germination or mycelium growth.

** spore did not germinate or mycelium did not grow.

Release of cellular constituents

As shown in Figure 1, the extracellular 260 nm absorbing material content of the experimental groups treated with DHA extract or DHA standard were significantly higher than that of the control group, indicating that the two drugs enhanced the cell permeability of spores. With the extension of treatment time, the OD₂₆₀ value was significantly increased ($P < 0.05$). The OD₂₆₀ value was significantly increased as the concentration of DHA extract increased from 1/2MIC to MIC and MFC ($P < 0.05$). MIC and MFC of DHA standard were both 4 mg/mL, so the OD₂₆₀ value of the two concentration treatment groups was ultimately similar, while the OD₂₆₀ value of the 1/2MIC treatment group was significantly lower than that of the MIC and MFC treatment groups ($P < 0.05$). The DHA extract or DHA standard can significantly improve the cell permeability of the spores, and the higher the concentration, the longer the treatment time, and the higher the cell permeability of the spores.

Cell membrane integrity

The cell membrane integrity of spores was detected by PI fluorescence staining assay. As shown in Fig. 2, the fluorescence intensity of the treatment group was significantly higher than that of the control group ($P < 0.05$), indicating that both drugs damaged the integrity of the cell membrane of *A. alternata* spore.

In DHA extract treatment group, the fluorescence intensity was significantly increased with the concentration from 1/2 MIC to MFC ($P < 0.05$). In the same concentration treatment group, the fluorescence intensity reached the maximum value after 0.5 h of treatment, and there was almost no change thereafter. In the DHA standard treatment group, due to the MIC and MFC were both 4 mg/mL, the fluorescence intensity of the corresponding two treatment groups was similar, while the fluorescence intensity of the 1/2MIC treatment group was significantly lower than that of the MIC and MFC treatment groups ($P < 0.05$). With the change of treatment time, the fluorescence intensity of MIC and MFC treatment groups reached the maximum value at 0.5 h, and that of 1/2MIC treatment group reached the maximum value at 1 h, and there was no significant change thereafter ($P > 0.05$). *A. Alternata* spore cell membrane will be damaged by DHA extract and DHA standard, and the higher the concentration of the drug used, the greater the damage.

ROS content

The content of ROS in *A. alternata* spores treated with different concentrations of DHA extract or DHA standard was detected by DCFH-DA fluorescent probe. As shown in Figure 3, in the DHA extract treatment group, ROS content of the 1/2 MIC treatment group reached the maximum value at 2 h, which was 2.63 ± 0.19 times of the initial ROS content. ROS content in MIC and MFC treatment groups reached the

maximum value at 1 h, which was 4.74 ± 0.23 and 12.36 ± 0.32 times of the initial ROS content, respectively, and then significantly decreased ($P < 0.05$). After treatment for 2 h, the ROS content decreased to 2.95 ± 0.33 and 10.53 ± 1.32 times of the initial ROS content, respectively, which may be caused by the leakage of ROS in spores fragmentation in later culture period. In the DHA standard treatment group, the ROS content of 1/2 MIC treatment group reached the maximum value at 2 h, which was 3.87 ± 0.57 times of the initial ROS content. ROS content of MIC and MFC treatment groups reached the maximum value at 0.5 h, which were 4.47 ± 0.47 and 4.45 ± 0.39 times of the initial ROS content, and had no significant change thereafter ($P > 0.05$).

MDA content

As shown in Fig. 4 a, the extracellular MDA content in DHA extract or DHA standard treatment group was significantly higher than that in the control group. In the DHA extract treatment group, the extracellular MDA content of the 1/2MIC, MIC and MFC treatment groups all increased with the treatment time, and increased to 1.49 ± 0.06 , 1.59 ± 0.04 and 1.71 ± 0.01 $\mu\text{M/g}$, respectively, after 2 h treatment. It was much higher than that of the control group (0.41 ± 0.06 $\mu\text{M/g}$). The MDA from *A. hedinii* leaves may remain in the DHA extract, leading to the detection of extracellular MDA in the DHA extract treatment group at 0 min. In the DHA standard treatment group, the extracellular MDA content of the 1/2MIC, MIC and MFC treatment group was 1.11 ± 0.05 , 1.24 ± 0.00 and 1.23 ± 0.04 $\mu\text{M/g}$, respectively, after 0.5 h treatment, and there was no significant change thereafter ($P > 0.05$).

As shown in Fig. 4 b, the intracellular MDA content in the DHA extract or DHA standard treatment group was significantly higher than that in the control group ($P < 0.05$), and the intracellular MDA content was much lower than the extracellular MDA content, which may be caused by the leakage of MDA from damaged cell membrane. In the DHA extract treatment group, the intracellular MDA content of 1/2MIC, MIC and MFC treatment groups reached 0.03 ± 0.00 , 0.03 ± 0.00 and 0.06 ± 0.01 $\mu\text{M/g}$, respectively, after 1 h treatment, and there was no significant change in the MIC treatment group thereafter ($P > 0.05$). The intracellular MDA content of 1/2MIC and MFC treatment groups decreased to 0.02 ± 0.00 and 0.03 ± 0.01 $\mu\text{M/g}$, respectively. In the DHA standard treatment group, the intracellular MDA content of 1/2MIC treatment group reached the highest value (0.07 ± 0.01 $\mu\text{M/g}$) at 2 h. In MIC and MFC treatment groups, the intracellular MDA content reached 0.06 ± 0.00 $\mu\text{M/g}$ at 0.5 h, then decreased gradually, and decreased to 0.04 ± 0.00 $\mu\text{M/g}$ at 2 h.

Activity of CAT

As shown in Fig. 5, the CAT activity of DHA extract and DHA standard treatment groups was much higher than that of control group at a certain time. In the DHA extract treatment group, the CAT activity of the 1/2MIC group reached 22.75 ± 0.50 U/g prot at 0.5 h, and there was no significant change thereafter ($P > 0.05$). CAT activity in MIC group reached 25.12 ± 1.37 U/g prot at 1 h, and then decreased to 22.05 ± 1.49 U/g prot at 2 h. CAT activity in MFC group reached 29.23 ± 1.73 U/g prot at 0.5 h, then decreased gradually,

and reached 11.55 ± 2.97 U/g prot at 2 h. In the DHA standard treatment group, the CAT activity of the 1/2MIC group increased gradually with treatment time, reaching 23.10 ± 1.49 U/g prot at 2 h. CAT activity in MIC and MFC groups reached the highest level at 1 h, which was 26.24 ± 1.50 U/g prot, and then decreased to 18.89 ± 0.00 U/g prot at 2 h.

The CAT activities of low concentration DHA extract and DHA standard treatment groups (1/2MIC extract, 1/2MIC DHA) were still high at 2 h, but the CAT activities of high concentration treatment groups (MIC extract, MFC extract, MIC DHA, MFC DHA) were decreased at 2 h. The MFC extract group was even lower than the control group. This was because the high concentration of drug caused more serious damage to the cell membrane of the spores after 2 h treatment, leading to apoptosis of some spores and inability to synthesize CAT.

Discussion

In this study, it was found that the DHA extract could effectively inhibit the germination of spores of *A. alternata*. The fluorescence intensity of PI staining and the absorbance of extracellular matter at 260 nm were significantly increased in the spores treated with DHA extract, which indicated that the DHA extract damaged the cell membrane of the spores. This result is consistent with previous studies (XU et al. 2018).

The oxidation of ROS is one of the antifungal mechanisms of many active substances (Zhang et al. 2015). In this study, there were significant changes in the content of ROS in each treatment group. The concentration of DHA extract ranges from 1/2 MIC to MFC, and the ROS content in the spores increased higher and higher. From 1 h to 2 h, the content of ROS in the MFC extract group decreased, which may be caused by the serious leakage of reactive oxygen in the damaged spores. The DHA extract promotes the production of ROS in the spores of *A. alternata*.

MDA is one of the final products of membrane peroxidation (Dou et al. 2017, Chen et al. 2019). High content of MDA indicates the occurrence of membrane peroxidation. In this study, MDA content changed significantly, extracellular MDA content did not show a downward trend, intracellular MDA content showed a downward trend in several groups (MFC extract, MIC DHA and MFC DHA), while total MDA content kept an upward or stable trend. It indicated that when the extract concentration was high and the treatment time was long, the spores were damaged more seriously, the intracellular MDA outflow increased.

CAT is one of the antioxidant enzymes, which are synthesized by cells to suppress the oxidation reaction (Liu et al. 2011, Wang et al. 2018). In this study, the CAT activity in spores treated with DHA extract for 0.5 h was relatively high, and it decreased to some extent after 2 h treatment. CAT activity in some treatment groups was even lower than that in the control group at 2 h, which may be caused by the serious damage of the spore membrane and many cell apoptosis after too long treatment. We also detected the activity of peroxidase (POD) and superoxide dismutase (SOD) in spores. POD and SOD activity in spores treated with DHA extract was 4–6 times higher than that in the control group. And no POD activity was detected in spores treated with DHA standard, it is possible that there are other

substances in DHA extract that can promote POD production in spores. The activities of CAT, SOD and POD in spores treated with the DHA extract suspension group were increased indirectly as a result of ROS level increase, which was a reaction to prevent peroxidation

In conclusion, this study reveals for the first time that DHA inhibits the spore germination of *A. alternata* by increasing the content of reactive oxygen species in spores, promoting membrane lipid peroxidation and destroying the cell membrane. This can be a reliable theory to enhance the quality and reliability of Antifungal biological pesticide.

Declarations

Acknowledgements

We thank Hubei Institute of Tobacco Science for providing the fungus strains and the raw materials of *Artemisia odoratum*.

Funding

This study was supported by the key science and technology project of Hubei Tobacco Co. (No. 027Y2018-009).

Conflict of interest We declare that there is no conflict of interest.

Ethical statement This research article does not contain any experiments/studies which require ethical clearance.

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Figures

Figure 1

Effects of DHA extract and DHA standard on the 260 nm absorbing material release

Figure 2

Effects of DHA extract and DHA standard on the integrity of spore cell membrane

Figure 3

Effects of DHA extract and DHA standard on the ROS content

Figure 4

Influence of DHA extract and DHA standard on MDA content

a. Extracellular MDA content; b. Intracellular MDA content; c. Total MDA content

Figure 5

Effects of DHA extract and DHA standard on CAT activity in spore