Study on the prevention and treatment of Aspergillus flavus pollution by Melaleuca alternifolia essential oil during the storage of Polygalae Radix Decoction Pieces

Wenxin Xia
School of Pharmacy, Ningxia Medical University
Lizhen Lin
School of Pharmacy, Ningxia Medical University
Jiahui Yue
School of Pharmacy, Ningxia Medical University
Yuxin Li
School of Pharmacy, Ningxia Medical University
Lulu Wang
School of Pharmacy, Ningxia Medical University
Yuanyuan Zhang
School of Pharmacy, Ningxia Medical University
Tingting Li
Key Laboratory of Ningxia Ethnomedicine Modernization, Ministry of Education, Ningxia Medical University
Lin Dong
School of Pharmacy, Ningxia Medical University
Xueyan Fu
xueyanfu2661@163.com

School of Pharmacy, Ningxia Medical University

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Abstract

In the natural environment, *Aspergillus flavus* often causes contamination of food, crop and Chinese medicinal materials, threatening the quality and safety of food, crop and medicinal materials and causing huge economic losses. In this study, we isolated, identified and screened the strains of *A. flavus* invading *polygalae radix*, and determined the changes of growth indexes, virulence, antioxidant system and cell membrane integrity of *A. flavus* under the treatment of *Melaleuca alternifolia* essential oil (MA-EO). Combined with the results of proteomics, the mechanism of action was clarified. In addition, this study also measured the change of active components in polygalae radix under the action of MA-EO. The results showed that MA-EO could damage cell membrane integrity, increase ROS accumulation, disrupt antioxidant system and inhibit the growth and toxicity of *A. flavus*. At the same time, MA-EO can significantly reduce the mildew of polygalae radix and curb the loss of active components after mildew.

1. Introduction

Aflatoxins (AFs), a class of toxic secondary metabolites with difuranocoumarin derivatives structure, are mainly produced by *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*). AFs that are more toxic include: Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), Aflatoxin G_2 (AFG_2) (Choochuay et al., 2018; Huertas-Pérez et al., 2018). Among them, AFB_1 is the most toxic, widely spread and commonly polluted, belonging to class I carcinogens, with carcinogenicity, teratogenicity and mutagenicity(Al-Zoreky and Saleh, 2019; DU et al., 2018). Agricultural crops such as wheat, corn, peanuts and so on are vulnerable to *A. flavus* pollution, so the prevention and control of *A. flavus* pollution of agricultural food crops has attracted wide attention(Shabeer et al., 2022). However, *A. flavus* pollution not only exists in agricultural food crops, but also exists in some medicinal and edible plants and Chinese herbal medicinal.

As an important part of traditional Chinese medicine (TCM), Chinese herbal medicine favored by the world for its unique curative effect, few adverse reactions and abundant sources. However, in recent years, the occurrence of excessive aflatoxin in TCM decoction pieces frequently affects the effectiveness and safety of TCM seriously. Wang *et al.*(Wang et al., 2013) tested 12 batches of licorice and 9 batches of fritillaria from different regions of China, and the results showed that 2 batches of licorice and 1 batch of fritillaria were positive. Yang *et al.*(Yu et al., 2019) detected AFs residue levels in 17 animal drugs, among which AFG_1 content was 1μg·kg^{-1} in bombyx batryticatus, and AFs and AFB_1 content was 6.4μg·kg^{-1} and 1.1μg·kg^{-1} in ground beetle, respectively. The production, processing, storage and transportation processes of Chinese medicinal materials are many and time-consuming. In addition, some medicinal materials contain much oil, high protein and high sugar content, so that Chinese medicinal materials are easy to be contaminated by *A. flavus* and cause mildew. In order to ensure the safety of Chinese herbal medicine, the World Health Organization and the European Union have established the limit standard of aflatoxin in Chinese herbal medicine (Trucksess et al., 2002; Who et al.,

1993). Chinese Pharmacopoeia (2020 edition) has clear limits on the total amount of AFB_1 and aflatoxin B_2, B_1, G_2, G_1 in some plant and animal medicines, and Polygalae Radix is one of them.

Polygalae Radix is the dry roots of *Polygala tenuifolia* Willd. or *Polygala sibiria* L. As one of the most commonly used Chinese medicinal materials, polygalae radix is in great demand for clinical use. It was first recorded in the "shennong herbal scripture" and has the effect of treating neurasthenia, palpitation, forgetfulness, insomnia and other diseases. In recent years, there have been many reports on aflatoxin pollution in polygalae radix, which is mainly produced in the process of planting, harvesting, processing and storage (Chien et al., 2018; Zhang et al., 2020). With the increasing attention to the safety of TCM, the reports about aflatoxins contaminated to TCM are increasing gradually (Zhang et al., 2017; Zhao et al., 2016). Due to the large differences in the distribution of aflatoxin residues in traditional Chinese medicine, and the difficulty in detection, it is of great significance to fully understand the pollution status, pollution routes and influencing factors of medicinal materials and related products, and to take effective measures to prevent and control them, so as to improve the safety of drugs and food and safeguard people's health.

In this study, we isolated, identified and screened the strains of *A. flavus* that invaded polygalae radix, and selected the strains with the highest amount of toxicity as the research object. By measuring the growth index, virulence, antioxidant system index and so on, we studied the inhibitory effect of tea tree essential oil on high-yield *A. flavus* strains and its mechanism. In addition, this study also measured the change of active components in polygalae radix under the action of Melaleuca alternifolia essential oil (MA-EO). The purpose of this study was to find out the relationship between plant essential oil in inhibiting the growth of Aspergillus flavus and the effective components in Radix polygalae, hoping that corresponding measures could be taken in the actual processing and storage process to reduce the risk of aspergillus flavus pollution and ensure the quality and safety of medicinal materials.

2. Material and Methods

2.1 Material and Reagents

Polgala tenuifolia Willd. was provided by Shanxi Academy of Agricultural Sciences and identified by Department of Traditional Chinese Medicine of Ningxia Medical University. *Melaleuca alternifolia* essential oil was obtained from Xue Song Natural Medicinal Oil Co., Ltd (Jiangxi, China). Potato Dextrose Agar (PDA) medium and Sabouraud Dextrose Broth (SDB) medium were purchased from Hope Biol-Technology Co., Ltd (Qingdao, China). 2,7-Dichlorodihydrofluorescein diacetate was purchased from Med Chem Express company. Catalase (CAT), glutathione peroxidase (GPX), total superoxide dismutase (T-SOD) and malondialdehyde (MDA) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). AFB1 ELISA rapid detection kit was purchased from Pribolab Bioengineering Co., Ltd (Qingdao, China). Tween-80, phosphate buffered saline (PBS), Propidium Iodide (PI) dye and other chemicals were purchased from Solarbio Science & Technology Co., Ltd (Beijing,

China). The membrane Protein Extraction Kit was purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China).

2.2 Extraction, isolation, purification and identification of *A. flavus*

Remove the soil and impurities from the moldy polygalae radix, then grind and sift. Weigh 1g, add 9mL ultra-pure water, put it in a centrifugal tube, and swirl it well. Put it in a 25 ℃ constant temperature culture oscillator, shake 150 r/min for 30 min, and let it stand for 5 min. Absorb the supernatant and add it to sterile water to make diluent with 10^{-2} concentration and 10^{-3} concentration, and store it in the refrigerator at -4℃ for future use.

The diluents (200μL) of the above concentrations were taken and coated in DG18 medium. The inoculated petri dishes were placed in a constant temperature incubator in the dark at 28±1℃ for 5d. After preliminary morphological observation, the colonies producing yellow spores on the appearance were selected and purified in DG18 medium until a single colony was obtained.

Single colony strains were selected and cultured on AFPA agar medium for observation, and then the orange transparent surface strains were selected for molecular biological identification. Total DNA of *A. flavus* was extracted by Solarbio fungal genome DNA extraction kit and the purity was detected. DNA sequences amplified by primers CL1 and CL2A were determined by agarose gel electrophoresis.

2.3 Determination of virulence of *A. flavus*

Spore suspensions of the selected *A. flavus* (1.0×10^{7}cfu/mL) were inoculated in 50ml of Sand's glucose liquid medium, and cultured at 28±1℃ for 5 days in a constant temperature shaker at 200r/min. AFB_1 content was determined by ELISA kit.

2.4 Determination of MA-EO effect on the growth of *A. flavus* and the biosynthesis of AFB_1

The strains of *A. flavus* were cultured in PDA medium for 120 hours at 29±1℃. The colony diameter was determined according to the modified agar plate method. The inhibition rate of radial growth (I) was calculated by the following formula, I% = (C-T) ×100 / C, where C represents the diameter of *A. flavus* colony in the control group, and T represents the diameter of *A. flavus* colony in the experimental group. After the culture of *A. flavus* in PDA medium for 120h, the spore suspension was prepared by adding 10 mL 0.05% tween-80 sterile solution into different culture dishes. The spore concentration was determined by hemocytometer under light microscope.

The spore suspension was inoculated into 50 mL SDB medium to make its final concentration 1×10^{5}cfu/mL. The appropriate amount of MA-EO was diluted to 0.1% (vol/vol), 0.2% (vol/vol) and 0.4% (vol/vol) with 0.05% (vol/vol) Tween-80 solution and the culture medium. Each concentration was repeated at least three times. *A. flavus* was incubated at 29±1 ℃ and 200 rpm for 120 h in the dark. Periodic sampling was performed at 24, 48, 72, 96 and 120h, respectively. *A. flavus* was filtered and

cleaned with Whatman No.4 filter paper in aseptic conditions, and the obtained mycelium was placed at 80 ℃ until constant weight, then weighed. AFB_1 determination method: The above culture medium was filtered by Whatman No.4 filter paper and determined by ELISA kit.

2.5 Effect of MA-EO on antioxidant enzyme activity and lipid peroxidation levels of *A. flavus*

A. flavus was cultured in SDB medium with 01%, 0.2%, and 0.4% (vol / vol) MA-EO. And, the control group had not MA-EO. The strains were cultured at 29 ± 1 ℃, 200 rpm and 120 hours in the dark. Obtained mycelia samples of *A. flavus* were filtered through two layers of gauze, and cleaned with sterile secondary water and 10 mmol/L PBS solution with pH 7.4. The treated equal weighted wet mycelia pullets were ground and homogenized, and the homogenizing solution was centrifuged at 4 ℃ and 12000g for 30min. Finally, the activities of CAT, T-SOD, GPX and MDA in the supernatant were measured by commercial kits.

2.6 Scanning electron microscopy and transmission electron microscopy assays

A. flavus mycelium was observed by scanning electron microscope (SEM). After washed with PBS, mycelia samples were fixed with 2.5% (vol / vol) glutaraldehyde solution at 4 ℃ for 2h, and washed with PBS buffer to remove free glutaraldehyde, then dehydrated with gradient alcohol (30%, 50%, 70%, 90%, 100%), dried at the critical point of carbon dioxide, coated with a layer of gold, and observed with SEM (S-3400N).

The ultrastructure was visualized for mycelia samples by Transmission electron microscope (TEM) following the similar procedure conducted. After washed with PBS, the mycelia pellets were fixed with 2.5% (vol / vol) glutaraldehyde solution and 4% (vol / vol) paraformaldehyde in PBS at 4 ℃, rinsed with PBS for 3 times, 10 minutes each, and then postfixed with 4% (w/v) osmic acid solution at 4 ℃ for 2h. Then, it was dehydrated with gradient alcohol (30%, 50%, 70%, 90%, 100%) and further embedded in Spurr resin at 65 ℃ for 16h. Then the mycelia ultrathin sections of about 50~70nm were obtained with an ultramicrotome, stained with 5% uranyl acetate and lead citrate. The stained portions were observed under TEM (HT7800).

2.7 Determination of cell membrane integrity

The mycelium was washed with sterile PBS buffer, then suction filtered, added with 10 ml of ice-cold 70% ethanol, fixed at 4℃ for 1h, and then washed with PBS twice, 5min each time. Five milliliters of PI staining solution at a concentration of 5μmol/L was added to EP tubes containing hyphae, mixed well, and incubated at 37 ℃ for 15min with shaking every 5min to ensure adequate mixing. The mycelium was taken and placed on a glass slide for observation by laser scanning confocal microscope (excitation light 545nm, emission light 590nm).

2.8 Proteomic analysis of membrane proteins

Fresh mycelium (100 mg) was collected, sliced and extracted using the cell membrane protein extraction kit. The obtained membrane protein solution was sent to Biomarker Technology Co., LTD. (Beijing, China) for identification and analysis. Lab-free proteomics was used for analysis, and the identified proteins were functionally annotated using GO and KEGG databases.

2.9 Treatment and culture of polygalae radix under the action of MA-EO

In this experiment, the "trans-culture" mode was adopted (after inoculation of *A. flavus* spore liquid on uncontaminated polygalae radix artificially) to investigate the changes of the active components in polygalae radix. The samples of polygalae radix were sterilized by ultraviolet irradiation, and 15g of polygalae radix were weighed and placed in sterilized glass conical bottles. The samples were sterilized by ultraviolet irradiation (265nm) for 5h, and then used. In this experiment, there were two groups, the normal group and the experimental group, with three samples in each group and three parallel samples in each sample. 0.5mL of *A. flavus* spore liquid was accurately absorbed and inoculated into the sterilized polygalae samples, and then 2mL sterile ultra-pure water was added. 100μL of tea tree essential oil was sucked into the filter paper, and the conical bottles were sealed with sealing membrane. The conical bottles were numbered and divided into normal tea tree essential oil group and tea tree essential oil experimental group. The conical bottles of the experimental group and the normal group were cultured in an artificial climate chamber with constant temperature and humidity (28±1℃, 80%RH) for 21 days. The samples of the normal group and the experimental group were taken out every 7 days, dried in an electric heating air drying oven, crushed in a high-speed crusher, through a No. 3 screen, placed in a zip lex-sealed bag, and stored in a refrigerator at -20℃.

2.10 Determination of the active components in moldy polygalae radix under the effect of MA-EO

This research adopts the high-performance liquid chromatography determination of Tenuifolin, Polygalaxanthone III and 3,6'-Disinapoylsucrose in mildewy polygalae radix. And the content of Tenuifolin, Polygalaxanthone III and 3,6'-Disinapoylsucrose was been detected after the treatment of MA-EO. The polygalae radix powder of the control group and the experimental group was precisely weighed, sieved, precisely weighed 1g, put into a conical bottle with stopper, precisely added 70% methanol solution (50mL), sonicated for 1h after weighed, weighed again after cooling, 70% methanol solution made up for the loss of weight, shake and filter. Precision amount of 25mL of filtrate, placed in a round-bottom flask to dry, add 50mL of 10% sodium hydroxide solution to the residue, heating reflux 2h, cooling, hydrochloric acid to adjust the pH value of 4~5, add water saturated n-butyl alcohol 50mL each time, and continuous shaking extraction 3 times, combined solution, After drying, the residue was dissolved with methanol and transferred to a 25mL volumetric flask. Methanol was added, the volume was fixed, and the sample solution was prepared. The sample solution was taken and passed through a 0.22μm filter membrane and set aside.

Respectively take Tenuifolin reference substance (batch number: 111849-202106, purity: 97.3%), Polygalaxanthone III reference substance (batch number: 111850-202006, purity: 95.3%) and 3,6'-Disinapoylsucrose reference substance (batch number: 111848-202006, purity, 96.5%) were precisely

weighed, methanol was added to make the reserve solution of reference substance, and then stored at 4℃ for later use.

Chromatographic conditions (Tenuifolin): Diamonsil Plus C_{18} (5μm 250×4.6mm); mobile phase: methanol (A) -0.05% phosphoric acid solution (B), injection ratio: A:B=70:30; Column temperature: 25℃; Flow rate: 1.0mL/min; Detection wavelength: 210nm; Injection volume: 10μL.

Chromatographic conditions (Polygalaxanthone III, 3,6'-Disinapoylsucrose): Diamonsil Plus C_{18} (5μm 250×4.6mm); Mobile phase: acetonitrile (A) -0.05% phosphoric acid solution (B), injection ratio: A:B= 18:82; Column temperature: 25℃; The flow rate was 1.0mL/min. Detection wavelength: 320nm; Injection volume: 10μL.

The methodological investigation and sample content determination were carried out under the above conditions.

2.11 Statistical analysis

SPSS 22.0 statistical software was used to process the experimental data, and Graphpad Prism 8.0 software was used to draw graphs. All experiments were measured in triplicate, and data were compared using one-way ANOVA.

3. Results

3.1 Isolation, purification, identification and AFB_1 determination of *A. flavus* from polygalae radix

Ten strains of fungi which could produce yellow spores were isolated from the contaminated polygalae radix by morphological observation. Colonies were further purified on DG18 medium until a single colony was obtained (FIG. 1a). The purified strains were inoculated on AFPA medium, and after 3 days of culture, it was observed that all the 10 strains could produce bright orange yellow colonies on the back of AFPA medium (FIG. 1b). The resulting DNA sequence was amplified using primers CL1 and CL2A, and the results of gel agarose electrophoresis are shown in FIG. 1c. All the 10 strains were confirmed to be *A. flavus* by PCR product purification and sequencing. The results showed that all the 10 strains could produce AFB_1, and the virulence of strain **1** was as high as 4.41 μg/mL (FIG. 1d). Strain **1** with the highest virulence was selected for subsequent experiments.

The toxicity of *A. flavus* in different hosts was significantly different. The dominant strains in peanut and cottonseed were high-yielding virulence strains, while the dominant strains in wheat and buckwheat were extremely low-yielding virulence strains (Duran et al., 2014; Zhou, 2020). This study showed that the dominant strain of polygalae radix was the high-yielding virulence strain. It is concluded that this may be related to the chemical composition of crops or medicinal materials, peanut, corn, cottonseed and polygalae radix contain more lipid components (Xi-mei et al., 2020).

3.2 MA-EO inhibited the growth of *A. flavus* and AFB_1 synthesis

A. flavus can form obvious colonies and produce a large number of spores on the surface of suitable solid medium. The present study showed that MA-EO inhibited the radial growth and colony formation of *A. flavus*. As shown in Figure 2a, after 24 hours of tea tree essential oil treatment, colonies could be seen in the control group, 0.1% MA-EO treatment group and 0.2% MA-EO treatment group, and the sizes of colonies were 1.3cm, 0.87cm and 0.6cm, respectively. After 48 hours of culture, no colony formation was found in 0.4% MA-EO treatment group, and there were significant differences among all groups ($P<0.05$). At the end of growth (120h), MA-EO showed a stronger concentration-effect relationship on the radial growth of *A. flavus.* At this time, the radial growth inhibition rates of 0.1%, 0.2% and 0.4% MA-EO were 31.77%, 51.56% and 75.52%, respectively.

The inhibitory effect of MA-EO treatment on the sporulation ability of *A. flavus* is shown in Figure 2b. After the whole culture period, the spore production of the tea tree essential oil treatment group was significantly different from that of the control group ($P<0.01$). The amount of spores produced by the control group was 1.73 times (0.1% MA-EO treatment), 2.51 times (0.2% MA-EO treatment) and 24.59 times (0.4% MA-EO treatment) higher than that of the experimental group, respectively, indicating that MA-EO could significantly inhibit the sporulation ability of *A. flavus*.

The inhibitory effect of MA-EO on *A. flavus* under liquid culture conditions is shown in Figure 2c. MA-EO has an obvious concentration-effect relationship on the formation and growth of *A. flavus* mycelium. In the experimental group, the higher the concentration of MA-EO, the better the inhibitory effect on mycelium formation and growth. After 72h incubation, the mycelium formation was completely inhibited by 0.4% MA-EO treatment.

Within 120h of culture, the dry weight of mycelia in the control group and the experimental group showed a positive correlation with time (Figure 2d). It increased to the maximum value at 120h, and there were significant differences among the groups at different culture stages ($P<0.05$). At the early stage of culture (24h), the dry weight of mycelia in the control group was 0.73g, and the dry weight of mycelia in the experimental groups was 0.59g (0.1% MA-EO treatment group), 0.44g (0.2% MA-EO treatment group) and 0g (0.4% MA-EO treatment group), respectively. At the end of culture (120h), the dry weight of mycelia in the control group was 1.45g, and the dry weight of mycelia in the experimental groups was 1.03g (0.1% MA-EO treatment group), 0.89g (0.2% MA-EO treatment group) and 0.001g (0.4% MA-EO treatment group), respectively. The growth of mycelium was completely inhibited in the high dose MA-EO treatment group during the whole culture stage.

The diameter of mycelium ball is an important parameter to measure the morphological characteristics of mycelium. In this study, the diameter of mycelium ball before and after treatment with MA-EO was measured. As shown in Figure 2e, the diameter of mycelium pellets in the control group showed a steadily increasing trend, and the diameter of mycelium pellets was 5.33 mm at 72 hours. At 120h after the experiment, the diameter of mycelium pellets in the experimental group reached the maximum. At this

time, the diameters of mycelium pellets in the control group and the 0.1% MA-EO treatment group were 5.50 mm and 5.33 mm, respectively, which were significantly higher than those in the 0.2% MA-EO treatment group (2.93mm) and the 0.4% MA-EO treatment group (0mm).

The amount of AFB_1 and AFT produced by MA-EO against *A. flavus* during different incubation stages under liquid culture conditions is shown in Figure 2f and 2g. The results showed that the accumulation of aflatoxin increased significantly with the extension of culture time. In the control group, the first 96 hours of growth was the rapid growth period of toxin production by *A. flavus*. At 96 hours, the content of AFB_1 was 3.82μg/mL, and the content of AFT was 7.61μg/mL . At 120 hours, the toxin production reached the peak. Compared with the control group, the peak of aflatoxin production of *A. flavus* in the MA-EO treatment group was from 96 to 120 hours. At 120 hours, the accumulation of aflatoxin in 0.1%, 0.2% and 0.4% MA-EO groups was 1.30μg/mL, 0.09μg/mL and 0.002μg/mL, respectively. The accumulation of AFT was 5.40μg/mL, 1.40μg/mL and 0.006μg/mL, respectively, which was significantly lower than that of the control group. After the whole culture period, the corresponding inhibition rates of different concentrations of MA-EO on the production of AFB_1 by *A. flavus* were 70.18% (0.1%), 97.94% (0.2%) and 99.95% (0.4%), respectively. The corresponding inhibition rates of MA-EO on AFT production by *A. flavus* were 30.5% (0.1%), 81.98% (0.2%) and 99.92% (0.4%), respectively.

3.3 Effects of MA-EO on morphological structure and ultrastructure of *A. flavus*

Scanning electron microscope observation showed that tea tree essential oil at different doses had effects on the organization and structure of *A. flavus* mycelium. The results of scanning electron microscopy (Figure 3a) showed that the mycelium in the control group was uniform in size, smooth in surface, and neatly arranged. Under the treatment of 0.1% MA-EO, the mycelium surface was rough, with many scale-like substances and arranged disorderly. When the concentration of MA-EO increased to 0.2%, the single mycelium was short and uneven, and the diameter of the tail of the mycelium became larger, with serious distortion. Transmission electron microscope observation showed that the hyphal organelle structure in the control group was intact, the matrix was uniform, the morphology was normal, the electron-dense layer of the cell wall was intact, and the mitochondria were smooth. In the 0.1% MA-EO group, the surface of mitochondria of hyphal cells was shrunken and the structure of cell wall was loose. The treatment with 0.2% MA-EO had seriously damaged the cell ultrastructure of the bacterial body, deformed the cell morphology, significantly reduced the number of mitochondria, thinned the thickness of the cell wall, changed the color of the intracellular matrix, and formed a large number of vesicles in the plasma membrane to form cavities (Figure 3b). It has been reported that plant essential oils have similar antibacterial properties and morphological changes against other harmful fungi (Li et al., 2013; Mitchell et al., 2016). It can be concluded that the antifungal effect of MA-EO may be partly attributed to the substantial damage of mycelial cell structure and organelles.

3.4 Effects of MA-EO on antioxidant system and reactive oxygen species (ROS) of *A. flavus*

The enzyme activities of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and the content of malondialdehyde (MDA) were measured by ELISA. During the whole cycle of cell culture, the MA-EO treatment group could significantly reduce the activity of CAT enzyme. The changing trend of CAT enzyme activity in *A. flavus* had obvious concentration-effect and time-effect relationship, that is, with the extension of culture time and the increase of concentration, the activity of CAT enzyme gradually decreased (Figure 4a). The effect of MA-EO on the GPx enzyme activity of *A. flavus* is shown in Figure 4b. At the initial stage of culture (48h), the GPx enzyme activity of the control group was 26.67 U/mg pro, which was not significantly different from that of the 0.1% MA-EO treatment group (29.43 U/mg pro) ($P<0.05$), compared with 0.2% MA-EO treatment group (44.15 U/mg pro), the difference was significant ($P<0.05$). With the extension of culture time, the GPx enzyme activity of *A. flavus* in the control group and the experimental group gradually increased, and reached the maximum at 120 hours. At the initial stage of culture, T-SOD activity in the control group (25.59 U/mg pro) was not significantly different from that in the 0.1% MA-EO treatment group (24.63U/mg pro) ($P<0.05$); When the concentration of MA-EO was increased to 0.2%, the T-SOD activity was significantly lower than that of the control group ($P<0.05$), which was 21.26 U/mg pro. At the end of the culture, the T-SOD activity in the experimental groups reached the lowest value. The 0.1% and 0.2% tea tree essential oil treatment groups were 13.25 U/mg pro and 12.91 U/mg pro, respectively. The T-SOD activity of the control group was 1.89 and 1.94 times higher than that of the 0.1% and 0.2% tea tree essential oil groups, respectively. The effect of MA-EO on malondialdehyde (MDA) content of *A. flavus* is shown in Figure 4d. The results showed that there was no significant difference in MDA content between the control group and the 0.1% MA-EO treatment group at the initial stage of culture ($P>0.05$), but the MDA content of 0.2% MA-EO treatment group was significantly higher than that of the other two groups ($P<0.01$). The above indicators showed that MA-EO treatment caused a great degree of disorder in the antioxidant system of *A. flavus* and caused strong oxidative damage to the bacteria.

The effects of MA-EO on the antioxidant system of *A. flavus* indicated that MA-EO had caused oxidative stress in *A. flavus*. We detected the intracellular ROS level of *A. flavus* at different culture stages by using the reactive oxygen species specific fluorescent dye DCFH-DA probe, and the experimental results are shown in Figure 5a. With the increase of tea tree essential oil concentration and the extension of culture time of *A. flavus*, the fluorescence intensity increased, that is, the intracellular ROS content of *A. flavus* increased. After 120 hours of culture, the relative fluorescence intensity of ROS in each group was 21.42 (control group), 66.50 (0.1% MA-EO treatment group) and 86.61 (0.2% MA-EO treatment group), respectively. With the increase of MA-EO concentration and the extension of action time, the green fluorescence intensity and fluorescence area increased significantly (Figure 5b), resulting in the increase of intracellular ROS level of *A. flavus* and the oxidative damage of bacterial cells.

3.5 Effect of MA-EO on cell membrane integrity of *A. flavus*

Due to the loss of membrane integrity of necrotic or damaged cells, PI dye can enter the cells and bind to DNA, so that the effect of MA-EO on the integrity of *A. flavus* cell membrane can be observed. As shown in Figure 6, the degree of damage to the integrity of the cell membrane of *A. flavus* by MA-EO showed a

positive correlation with the concentration of MA-EO and its action time. The results showed that tea tree essential oil could change the membrane permeability of *A. flavus*, destroy the integrity of the cell membrane, and then cause cell death.

3.6 Effects of MA-EO on membrane protein of *A. flavus*

A total of 1604 membrane proteins of *A. flavus* were identified in our previous study (Lin et al., 2023). Through proteomic analysis, we further studied the changes of cell membrane protein levels during the inhibition of *A. flavus* growth by MA-EO. According to the comparison results in Fig.7a and 83 membrane proteins were destroyed and 179 intracellular proteins were released by MA-EO. As shown in Fig.7b, in the GO analysis of destroyed membrane proteins, 123, 55 and 83 proteins were classified as biological processes, cellular components and molecular functions respectively. In the classification of biological processes, metabolic process accounted for the highest proportion, followed by cellular process and single-organism process. In cellular component classification, it is mainly enriched in cell, cell part, macromolecular complex, membrane part and organelle. In molecular function classification, it is mainly enriched in antioxidant activity, binding and catalytic activity. To understand the pathways involved in these proteins, we performed KEEG analysis. As shown in Fig.7c, the number of proteins involved in the metabolic process is the largest and the main pathways involved include arginine biosynthesis, tryptophan metabolism, aflatoxin biosynthesis, fructose and mannose metabolism, etc.

In the GO analysis of released intracellular proteins, the results of Fig.7d showed that 263, 252 and 157 proteins were classified as biological processes, cellular components and molecular functions, respectively. The metabolic process, cellular process and single-organism process are the biological processes with the most abundant genes. As shown in Fig.7e, most proteins can be determined to be involved in metabolism, genetic information process and cellular process through pathway analysis. A few proteins are involved in environmental information processing and human disease pathways.

These results suggested that MA-EO may exert its anti-*A. flavus* effect mainly through the destruction of membrane proteins involved in metabolic and genetic information processes.

3.7 Effect of MA-EO on the active components of mildew polygalae radix

Tenuifolin, Polygalaxanthone III and 3,6'-Disinapoylsucrose is quality evaluation indexes of polygalae radix. This experiment by HPLC determined the content of tenuifolin, polygalaxanthone III and 3,6'-Disinapoylsucrose in mildew polygalae radix. In the study, polygalae radix in the process of trans-culture, contamination by *A. flavus* can significantly reduce the content of tenuifolin, polygalaxanthone III and 3,6'-Disinapoylsucrose in polygalae radix. After 21 days of trans-culture, the content of tenuifolin in the infected polygala decreased by 20.88% compared with the control group, showing a significant difference . There was no significant change in the content of tenuifolin in the normal MA-EO group compared with the control group. Compared with the other two groups, the content of tenuifolin in the MA-EO experimental group fluctuated slightly after 7 days of trans-culture, and there was no significant difference($p>0.05$) (Fig.8a). The content of polygalaxanthone III and 3,6'-Disinapoylsucrose in mildew

polygalae radix were significantly lower ($P<0.05$) after trans-culture. Compared with control group, the content of polygalaxanthone III was reduced by 9.53%, 3, 6-2 mustard acyl sucrose was reduced by 7.73%. Under the treatment of MA-EO, the content of polygalaxanthone III and 3,6'-Disinapoylsucrose in polygalae radix was no significant change (Fig.8b and 8c). In conclusion, MA-EO treatment has a protective effect on the effective components of Polygalae Radix, which can effectively reduce the loss of effective components.

4. Discussion and Conclusion

Mycotoxins is one of the main aspects of exogenous harmful residues contamination of TCM, which is an important factor affecting the quality and safety of TCM. The improper control and management in the whole industrial chain of TCM, such as cultivation, breeding, processing, production, storage and circulation, may lead to mycotoxin pollution, and bring safety risks to the original medicinal materials, decoction pieces and related preparations. Among them, because most Chinese herbal decoction pieces are processed slices, the damaged parts are vulnerable to invasion by external microorganisms, which will seriously affect their quality. As one of the dominant fungi in the contamination of TCM materials, Aspergillus has attracted much attention. *A. flavus* has a wide host range and can infect a variety of medicinal plants such as polygalae radix, jujube kernel, peach kernel and lotus seed. The study showed that the Chinese medicinal materials susceptible to aflatoxin contamination involved more than 20 kinds of subclasses, more than 10 kinds of roots and rhizome, including polygalae radix, licorice, rhubarb, salvia miltiorrhiza and other clinical commonly used bulk medicinal materials (Zhenying and Yongqing, 2021). In addition, in a total of 21 batches of *polygalae* radix samples from 2017 to 2018, the unqualified rate of aflatoxin content was as high as 90.5% (http://samr.cfda.gov.cn/WS01/CL0001/). The excessive aflatoxin in *polygalae* radix causes a large amount of waste, which makes the shortage of medicinal resources of *polygalae* radix worse in our country. At present, the methods to inhibit the rise of aflatoxin in TCM mainly include physical and chemical methods, which slow down the growth rate or reduce the content of aflatoxin to a certain extent. In recent years, fumigation of traditional Chinese medicinal materials with plant essential oil to inhibit the growth of *A. flavus* is an effective way to inhibit the content of aflatoxin in traditional Chinese medicinal materials.

Plant essential oils are secondary metabolites produced by plants with complex compositions, containing dozens or even hundreds of ingredients, and are known for their antibacterial, antifungal, insecticidal and antiviral properties. Now, the antibacterial mechanism of plant essential oil can be divided into the following four categories: (a) destroying the protective structure of bacteria in vitro and enhancing cell permeability; (b) destroying the respiratory and metabolic systems and cause energy metabolism disorder; (c) hindering the synthesis of nucleic acid and protein and affecting cell structure and function; (d) inhibiting the production of bacteria and conidia, block the generation of pathogen progeny, so that the bacteria population gradually decline (Cai et al., 2022; Tariq et al., 2019). This study showed that MA-EO can inhibit the growth and toxicity of *A. flavus* by damaging cell membrane integrity, increasing ROS accumulation, disrupting antioxidant system, causing mitochondrial disorders of bacterial somatic cells, and substantial damage to cell structure and organelles. Plant essential oil is a safe, natural, pollution-

free and excellent plant product, with a broad spectrum of bactericidal effect, not only widely used in food, medicine and other aspects, but also in the field of industrial mildew prevention has been widely studied. In this study, MA-EO was determined to have a strong inhibitory effect on *A. flavus* and its mechanism was explored. On this basis, the changes of the index components of MA-EO in the mildew control application of polygalae radix were investigated. The results showed that the infection of *A. flavus* could significantly reduce the active components of polygalae radix, and the intervention of MA-EO could significantly reduce the mildew of polygalae radix, and had no effect on the content of the index components. These results indicated that plant essential oil can be used as an important strategy to prevent mildew in Chinese medicinal materials.

This study found that *A. flavus* infection can significantly reduce the content of tenuifolin, polygalaxanthone III and 3,6'-Disinapoylsucrose. After treatment with MA-EO, the mildew phenomenon was reduced and the loss of active components caused by mildew was prevented effectively. MA-EO can significantly inhibit the growth and toxin production of *A. flavus*, and its mechanism is mainly related to early cell apoptosis accompanied by mitochondrial dysfunction, cellular oxidative stress and cell membrane damage.

Declarations

CRediT authorship contribution statement

Wenxin Xia: Methodology, Writing - original draft, Writing - review & editing, Supervision. **Lizhen Lin:** Methodology, Investigation, Resources, Validation. **Jiahui Yue, YuxinLi, LuluWang:** Methodology, Resources, Visualization. **Yuanyuan Zhang, Tingting Li:** Software, Conceptualization, Methodology. **Lin Dong:** Resources, Methodology. **Xueyan Fu:** Methodology, Writing - review & editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

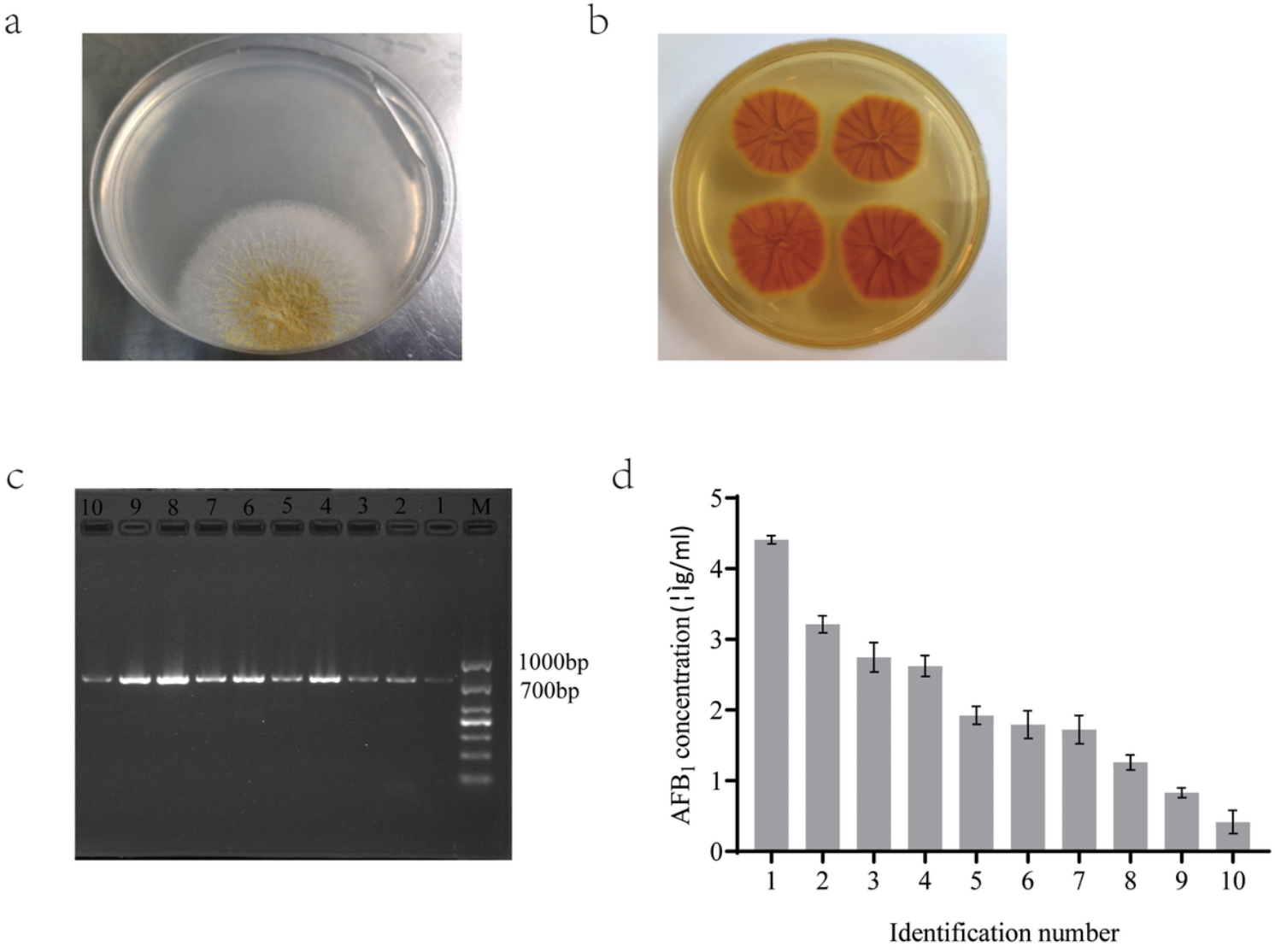

Figure 1

(a) A single strain of *A. flavus*; (b) Colony morphology of *A. flavus*on AFPA; (c) PCR sequence amplification results of *A. flavus* in Polygalae radix. M: DL1000 DNA marker; 1-10: The product of PCR. (d) AFB1 concentrations for *A. flavus* in *Polygala radix* ($\bar{x}$ ±s n=3).

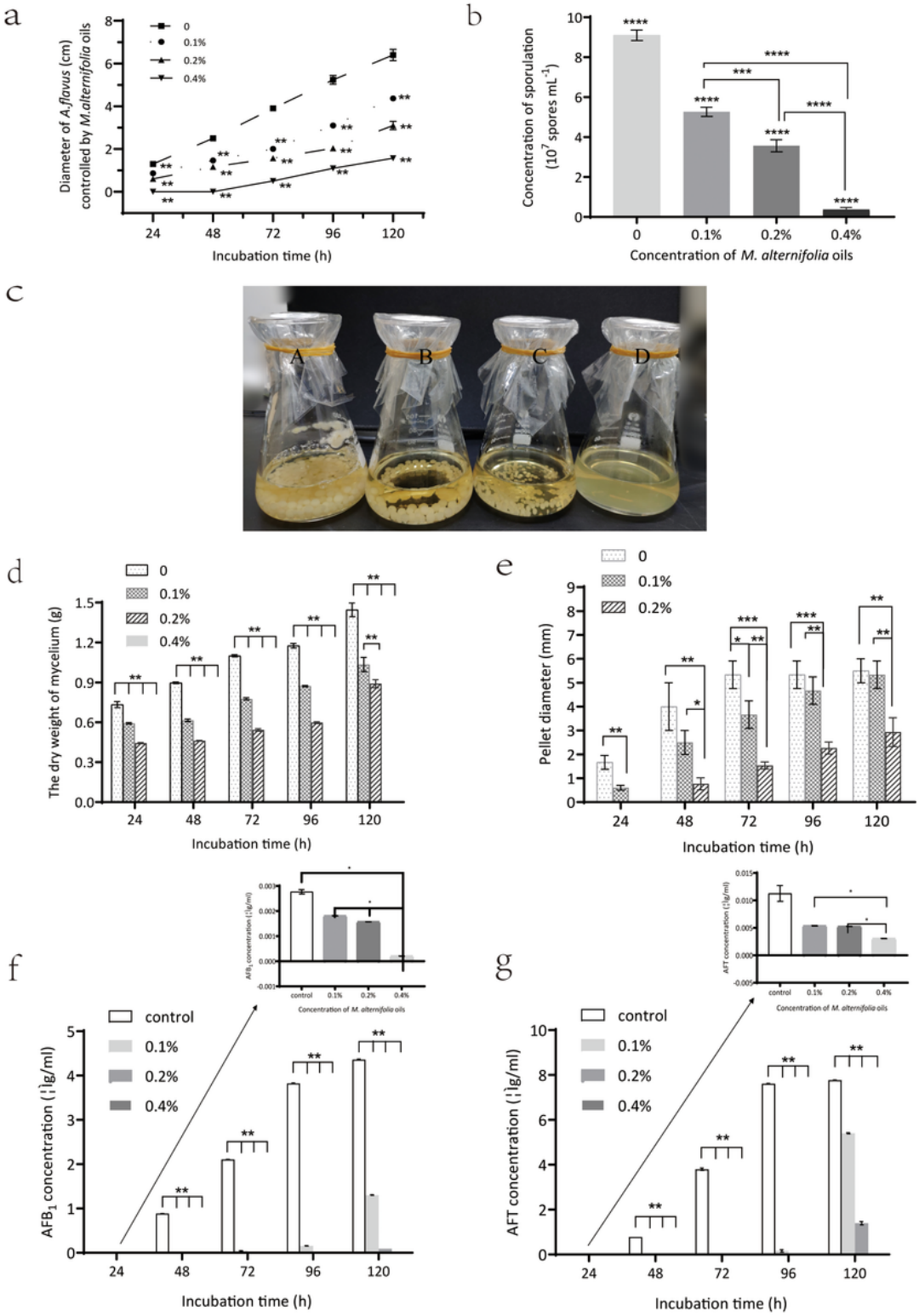

Figure 2

(a) Effect of the MA-EO treatment on *A. flavus* colony growth during 5 days of incubation. (b) Effect of the MA-EO on spore production of *A. flavus.* (c) Effect of different MA-EO on growth of *A. flavus* at 72h of incubation period, including (A) 0%, (B)0.1%, (C)0.2%, (D)0.4%. (d) Effect of different MA-EO treatment on mycelium weigh. (e) Effect of MA-EO on mycelial pellet diameter of *A. flavus*. (f) Effect of the MA-EO on the concentration of AFB_1 produced by *A. flavus* in 5-day cultures. (g) Effect of the MA-EO on the

concentration of AFT produced by *A. flavus* in 5-day cultures. ($\bar{x}$ ±s, n=3; Compared with control *P< 0.05, **P< 0.01, ***P< 0.001)

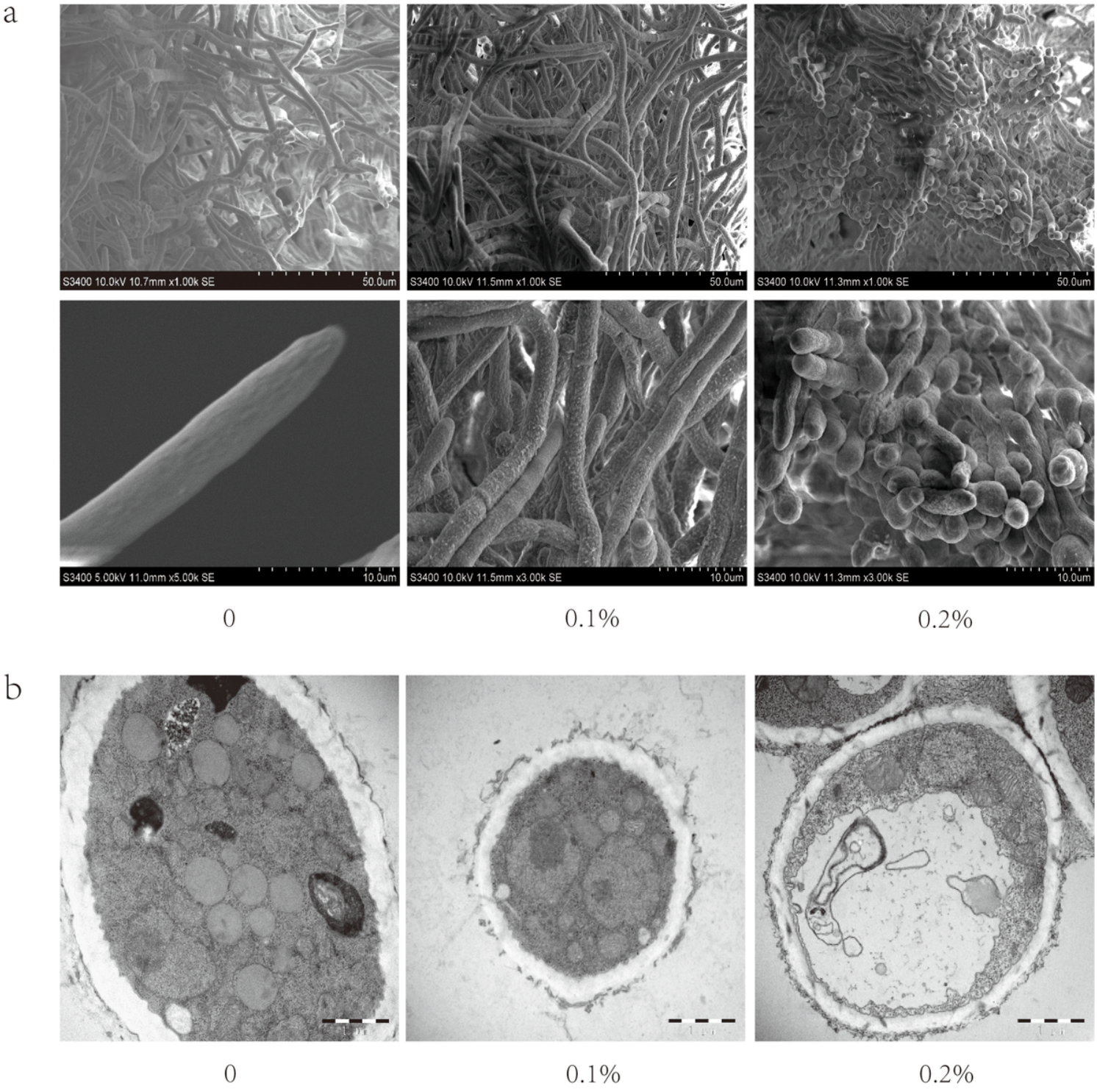

Figure 3

(a) Effect of MA-EO on the mycelium tissue and structure from *A. flavus.* (b) Effect of MA-EO on the hyphal ultrastructure of *A. flavus*.

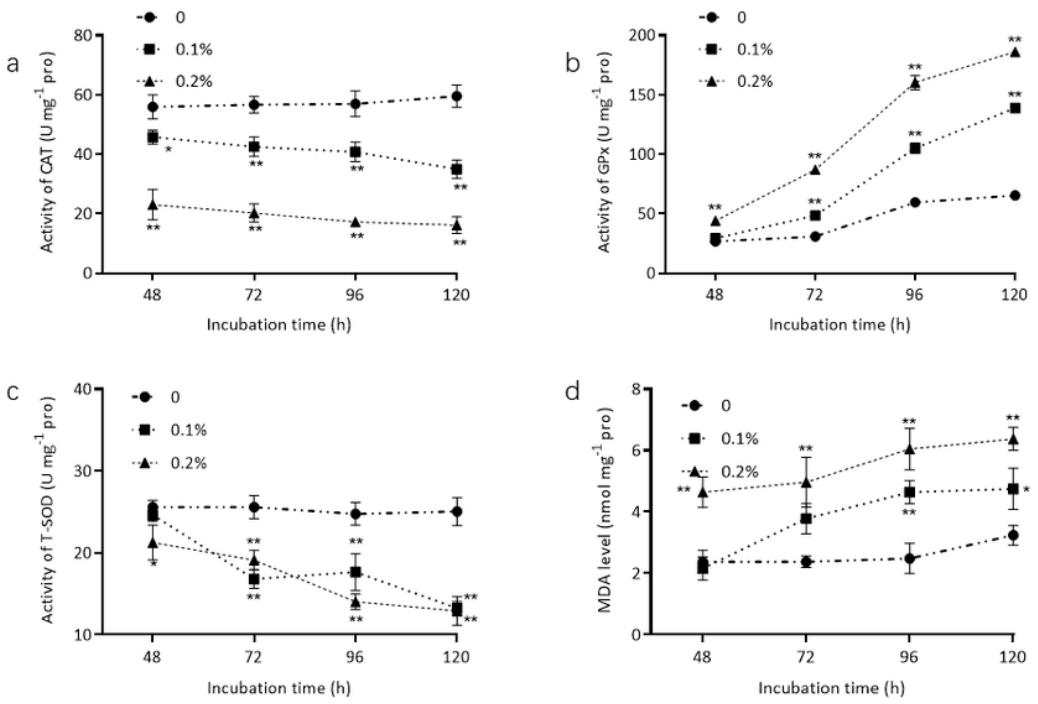

Figure 4

Effect of MA-EO treatment on the content of CAT, GPx, T-SOD and MDA from *A. flavus*. ($\bar{x}$ ±s, n=3; Compared with control *P< 0.05, **P< 0.01, ***P< 0.001)

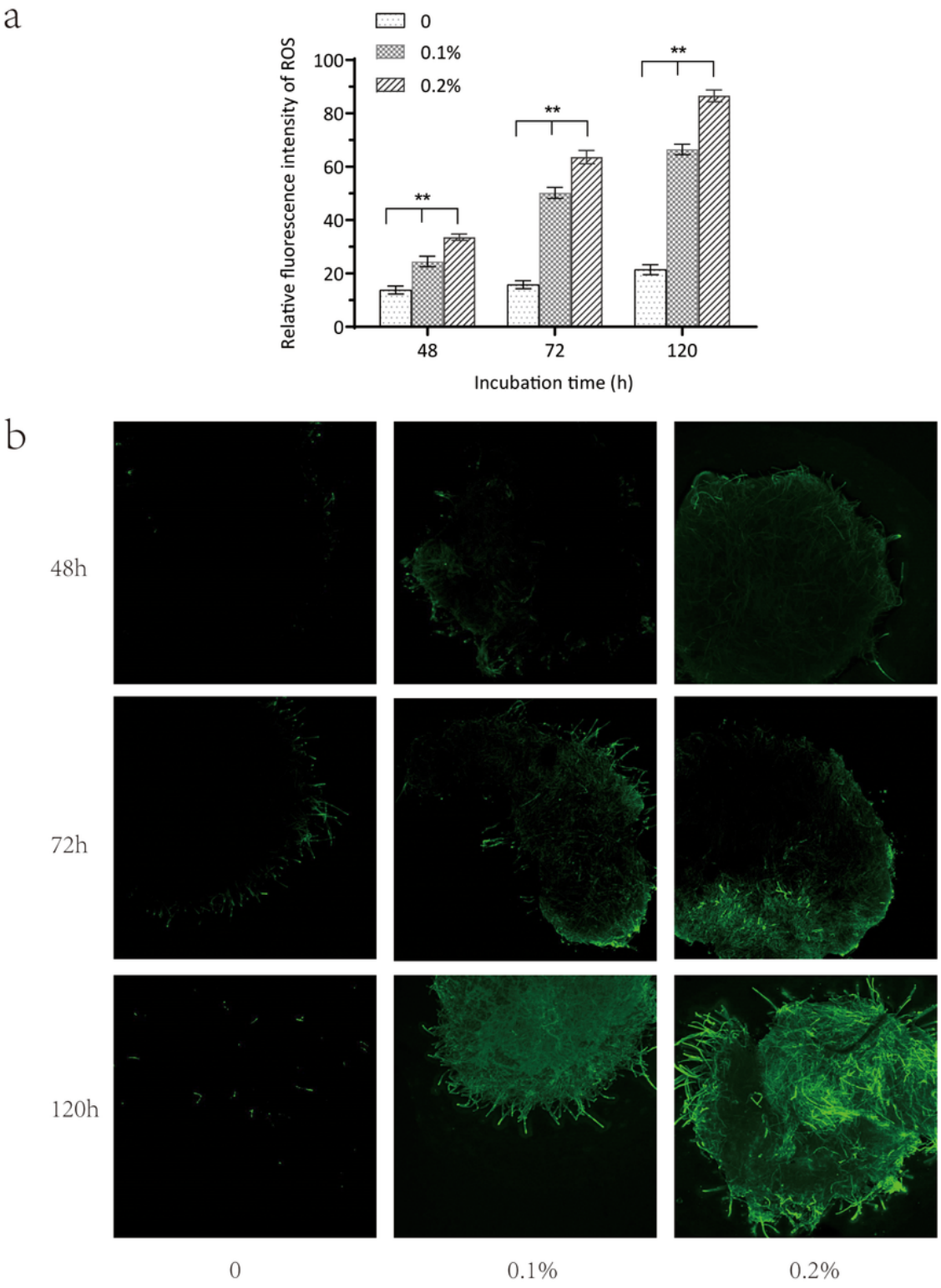

Figure 5

Effects of MA-EO on the ROS of *A. flavus*.

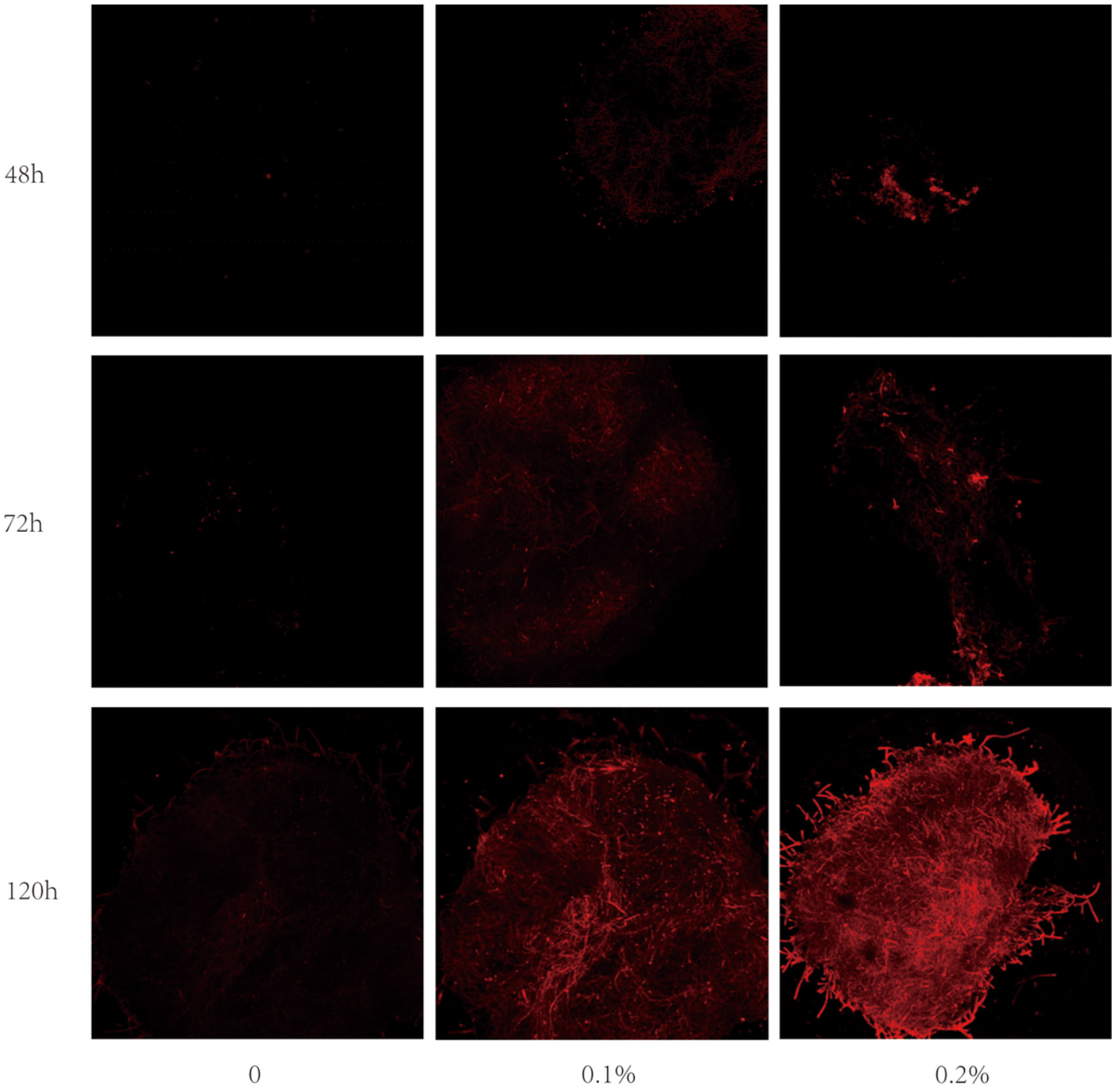

Figure 6

Effect of MA-EO on cell membrane permeability of *A. flavus*.

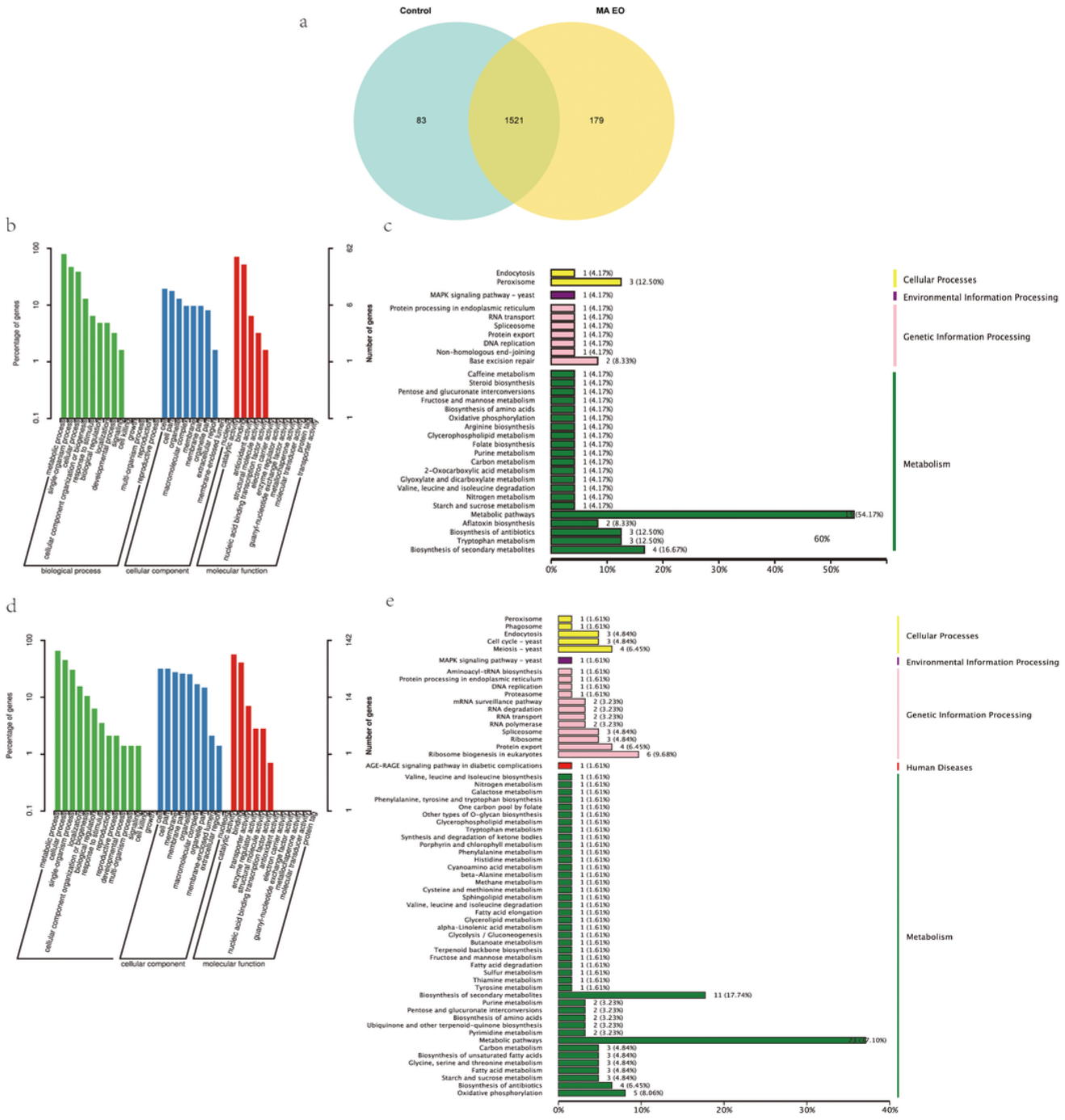

Figure 7

Venn diagram of membrane protein number in control group and MA-EO treatment group (a). Analysis and identification of membrane proteins altered by MA-EO treatment, GO analysis of 83 destroyed membrane proteins (b), KEEG analysis of 58 destroyed membrane proteins (c), GO analysis of 179 newly released membrane proteins (d), , KEEG analysis of 179 newly released membrane proteins (e).

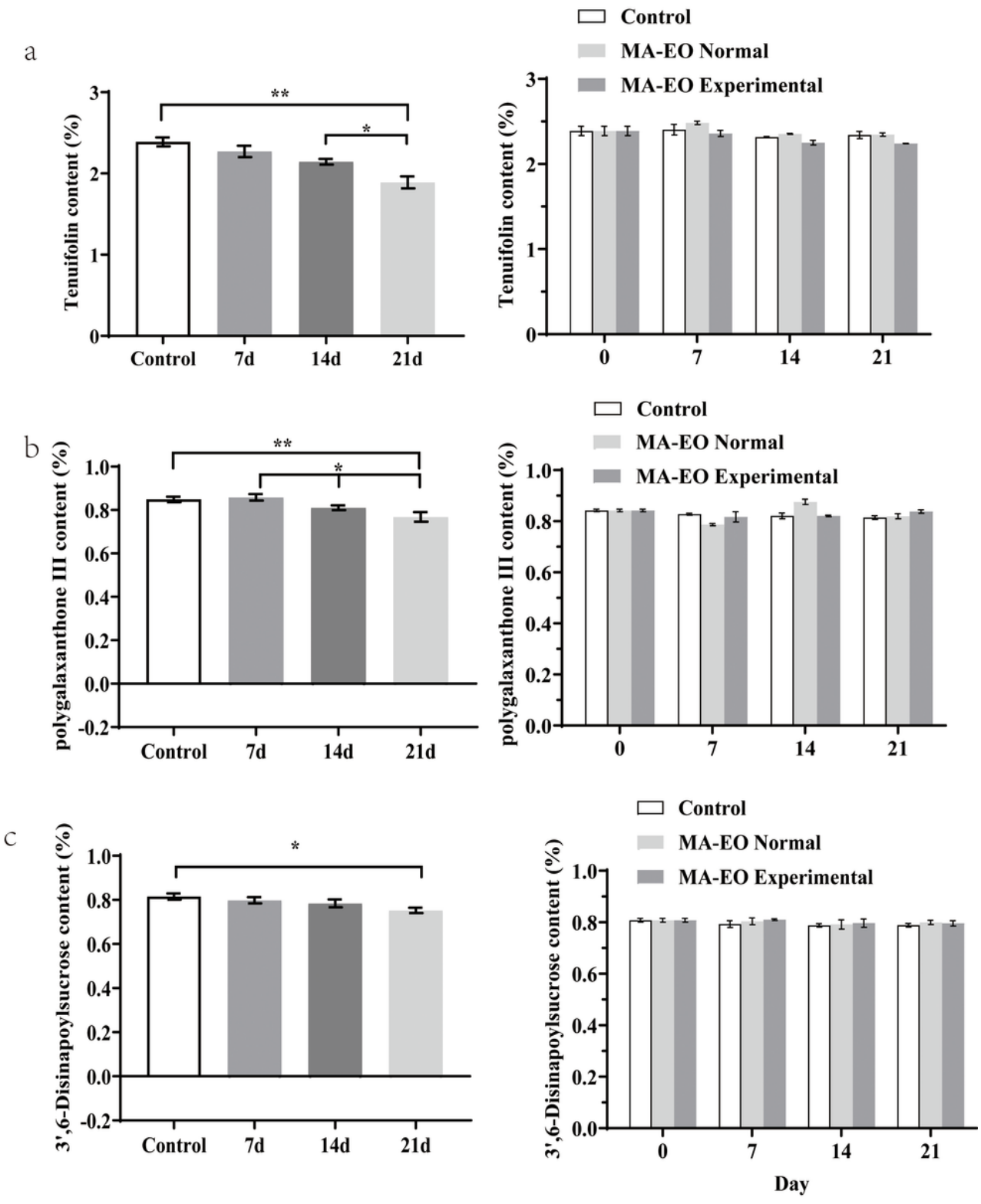

Figure 8

(a) Tenuifolin content determination results under the condition of *A. flavus* (left), Effect of the MA-EO on Tenuifolin content (right); (b) polygalaxanthone III content determination results under the condition of *A. flavus* (left), Effect of the MA-EO on polygalaxanthone III content (right); (c) 3',6-Disinapoylsucrose content determination results under the condition of *A. flavus* (left), Effect of the MA-EO on 3',6-Disinapoylsucrose content (right). ($\bar{x}$±s, n=3; Compared with control *P<0.05, **P<0.01)