



Influence of flavonoids from *Sedum aizoon* L. on mitochondrial function of *Rhizopus nigricans* in strawberry

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Received: 31 October 2023 / Accepted: 22 March 2024 / Published online: 13 April 2024
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Abstract

Rhizopus nigricans (*R. nigricans*), one of the fungi that grows the fastest, is frequently discovered in postharvest fruits, it's the main pathogen of strawberry root rot. Flavonoids in *Sedum aizoon* L. (FSAL) is a kind of green and safe natural substance extracted from *Sedum aizoon* L. which has antifungal activity. In this study, the minimum inhibitory concentration (MIC) of FSAL on *R. nigricans* and cell apoptosis tests were studied to explore the inhibitory effect of FSAL on *R. nigricans*. The effects of FSAL on mitochondria of *R. nigricans* were investigated through the changes of mitochondrial permeability transition pore(mPTP), mitochondrial membrane potential(MMP), Ca²⁺ content, H₂O₂ content, cytochrome *c* (*Cyt c*) content, the related enzyme activity and related genes of mitochondria. The results showed that the MIC of FSAL on *R. nigricans* was 1.800 mg/mL, with the addition of FSAL (1.800 mg/mL), the mPTP openness of *R. nigricans* increased and the MMP reduced. Resulting in an increase in Ca²⁺ content, accumulation of H₂O₂ content and decrease of *Cyt c* content, the activity of related enzymes was inhibited and related genes were up-regulated (*VDAC1*, *ANT*) or down-regulated (*SDHA*, *NOX2*). This suggests that FSAL may achieve the inhibitory effect of fungi by damaging mitochondria, thereby realizing the postharvest freshness preservation of strawberries. This lays the foundation for the development of a new plant-derived antimicrobial agent.

Keywords Flavonoids from *Sedum aizoon* L. (FSAL) · *Rhizopus nigricans* · Antifungal mechanisms · Mitochondria

Introduction

Widespread plant pathogenic fungi jeopardize fruit production and safety while also inflicting significant economic losses. Plant (Oztekin et al. 2023). *R. nigricans*, one of the fungi that grows the fastest, is frequently discovered in postharvest fruits, it's the main pathogen of strawberry root rot (Abied et al. 2023). Fungicides are commonly used to prevent the spread of pathogenic fungi, but can make fungi resistant and negatively impact human health and the environment (Nunes 2011). The creation of a number of effective

and safe fungicides with innovative structures, distinctive modes of action, and no cross-resistance is therefore urgent.

Flavonoids, usually derived from plants, have antioxidant (Ganeshpurkar and Saluja 2019), anti-inflammatory (Naeem et al. 2022) and other properties (Seo et al. 2016). In addition, flavonoids also have antifungal and antibacterial activities. *Sedum aizoon* L. is a wild herb, which rich in bioactive components (Xu et al. 2015). Flavonoid from *Sedum aizoon* L. (FSAL) is an oily liquid with special aroma extracted from *Sedum aizoon* L. The major components of this purified flavonoid sample, quercetin and kaempferol (Xu et al. 2019). It has been proved that quercetin affects the mitochondrial dysfunction of *Candida albicans*, causing the disruption of the entire antioxidant reduction system of the bacterium, ultimately leading to apoptosis (Kwun and Lee 2020), and kaempferol mainly exerted bacteriostatic effects at the molecular level by affecting bacterial energy metabolism, reducing pathogenicity, and leading to disruption of cellular integrity, leakage of contents and cell death eventually (Li et al. 2022). It has been proved that flavonoids from *Sedum aizoon* L., as a natural preservative, can effectively

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inhibit the deterioration of grapes caused by pathogenic microorganisms after harvest (Wang et al. 2023), and has the effect of preserving the freshness of grapes and *Penaeus vannamei* (Wang et al. 2020). Therefore, based on the excellent bacteriostatic and safety performance of FSAL, this experiment discussed the bacteriostatic effect and mechanism of FSAL on *R. nigricans*, which provided a theoretical justification for the management of FSAL on *R. nigricans*, and also provided a certain fresh-keeping method for strawberry post-harvest storage.

Mitochondria is an important place for energy metabolism of fungi, as well as a core organelle for respiration, which plays an important role in the process of apoptosis. Many researchers have studied the characteristics of fungal cell apoptosis, which is similar to cell apoptosis in mammalian cells, with the release of *Cyt c*, Ca^{2+} accumulation, phosphatidylserine (PS) exposure, and the opening of mPTP pores observed in fungal cells undergoing apoptosis (Madedo et al. 2004). Therefore, we investigated several features of fungal apoptosis and characterized a novel fungicidal mechanism of FSAL.

The aim of this study was to understand the mechanism of the antifungal activity of FSAL. The data obtained in this study showed that the extract showed a strong effect on mitochondrial function, so mitochondrial function was the main target.

Materials and methods

Plant materials and treatment

Strawberries are picked in February at the Da Niushan Picking Garden in Ningbo, China. And the strawberries were transported to the laboratory immediately after picking, the fruits with consistent maturity, uniform size, uniform color, no mechanical damage, and free of pests and diseases were selected for the test.

According to the study (Luo et al. 2020), FSAL was collected of *Sedum aizoon* L. were attained in Peixian County (Jiangsu, China), flavonoids are mostly made from the leaves and stems of this plant. After drying, they were ground into a powder. The substance was temporarily extracted with ethanol by rotary evaporation. The crude flavonoid extract was purified using AB-8 macroporous resin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) following rotary evaporation. The purified sample was stored at $-80\text{ }^{\circ}\text{C}$ before to use.

Culture of fungal strain

The China Industrial Microbial Strain Conservation and Management Center provided *R. nigricans* (CICC, 41,346).

R. nigricans spore suspension preparation

R. nigricans was inoculated into PDA plate medium for activation, and cultured at $25\text{ }^{\circ}\text{C}$ for 7 d, and then reactivated under the same conditions for reserve. Sterile water (containing 0.5% Tween 80) was washed and the spores were scraped off the plate. The spores were collected and filtered with 4 layers of sterile gauze (Shanghai Yongchuan Biotechnology Co., LTD., Shanghai, China) to remove mycelium. The spore count was adjusted to 1×10^6 spores / mL using a blood cell counting plate.

Preparation of *R. Nigricans* mycelia suspensions

The potato glucose broth (PDB) medium was supplemented with the *R. nigricans* spore suspension, cultured for 48 h on a rotary shaker at $28\text{ }^{\circ}\text{C}$ and 120 rpm, and the mycelium was obtained by filtration with four layers of gauze.

Determination of strawberry fruit disease incidence

After selection, the fruits were rinsed, the surface was dried, and then a wound of 3×3 mm was stabbed at the equatorial part of the fruit with a sterilized lance tip, and 20 μL of FSAL (at concentrations of 0.45, 0.9, and 1.8 mg/mL) and aseptic water were injected into the wound, respectively, and then the wound was dried naturally, and then 10 μL of 10^4 spore/mL of *R. nigricans* spore suspension was accessed at the wound. After drying, the fruit was transferred into a plastic preservation box and incubated at room temperature $25\text{ }^{\circ}\text{C}$ and relative humidity (85%). Incubation was carried out until the 30 h to measure the incidence.

The disease incidence was presented as the percentage of decay fruits with respect to the total number of fruits. The incidence of fruits was counted, 10 strawberry fruits were set up for each treatment and the experiment was replicated at least 3 times.

Determination of the minimum inhibitory concentration (MIC)

FSAL was mixed with PDA medium by half dilution method to prepare FSAL mixture of different concentrations (0.000, 0.225, 0.450, 0.900, 1.800, 3.600, 7.200, 14.400 mg/mL), and poured into a plate to cool for use. Control tablets did not contain phenanthrene flavones. 20 μL spore suspension with a concentration of 10^6 spores/mL was added and

uniformly coated. The culture was carried out at 25 °C for 2 d with 5 plates in each group and repeated three times. The lowest flavonoid concentration with no mycelial growth was used as the MIC.

Determination of apoptosis

The fungi suspension that grew to the logarithmic stage was added to FSAL so that its final concentration reached 1.0 MIC, and the same volume of PBS was added as the control group. They were thoroughly mixed and cultured at 25 °C. The fungi solution was obtained at 0 h, 4 h and 8 h respectively, washed three times with pre-cooled PBS, centrifuged at 1000 g for 5 min according to the Annexin V-FITC/PI apoptosis kit instructions (Shanghai Biyuntian Biotechnology Co., LTD), and the supernatant was discarded to collect cells. The cells were gently suspended with PBS. Centrifuge, discard the supernatant, gently resuspension the cells with 195 ul Annexin V-FITC binding solution, and gently mix with 5ul Annexin V-FITC. Add 10 ul PI, mix well, incubate at room temperature (20–25 °C) for 10–20 min away from light, place in an ice bath. The apoptosis rate was detected by confocal laser after incubation.

Extraction of mitochondria

Refer to the method of Zheng et al. (Zheng et al. 2015) to extract mitochondria from *R. nigricans*. The filaments of *R. nigricans* were treated with 0.00 and 1.800 mg/mL of FSAL for 0, 4, and 8 h, filtered and washed three times with frozen PBS, ground with liquid nitrogen, and then mixed with the extract. For 10 min, the homogenate was centrifuged at 4000 g by differential centrifugal method, and the supernatant was centrifuged at 10,000 g for 15 min. The precipitate is re-suspended in the extraction medium and stored at 4 °C for subsequent steps. The Coomassie bright blue assay kit was used to determine the protein content in mitochondria.

Determination of mitochondrial membrane potential (MMP)

The spore suspension of *R. nigricans* was collected and suspended in 0.01 M PBS (pH 7.2), and the flavonoid was added to the final concentration of 0.00 and 1.800 mg/mL. After incubating at 25 °C for 4 h, the spores were collected and washed with pre-cooled PBS, and incubated with fluorescent probe JC-1 in an incubator at 37 °C for 20 min. Then, the spores were washed three times and then suspended in the stain buffer, and mitochondrial membrane potential (MMP) was detected by flow cytometry.

Determination of mitochondrial permeability transition pore (mPTP)

The spore suspension of *R. nigricans* was collected and suspended in 0.01 M PBS (pH 7.2), and FSAL was added until the final concentrations were 1.800 mg/mL, the control group was treated with pure water. Spores were collected after incubation at 28 °C for 0 h, 4 h and 8 h. The mPTP openness of *R. nigricans* was measured using the Mitochondrial Permeability Transition Pore Assay Kit (biosharp Corporation) according to the manufacturer's instructions, and detected by flow cytometry.

The green fluorescence signal of Calcein was detected by flow cytometry, and the degree of mPTP openness was determined by comparing the intensity of the fluorescence signal, and then the degree of mitochondrial damage was inferred.

Determination of Ca²⁺ content

Reference Kim et al. (Kim et al. 2020) methods were modified. Fluo-3 AM was used to detect intracellular calcium ion concentration at 488 nm excitation wavelength and 525 nm emission wavelength. The suspensions grown to logarithmic stage were collected by centrifugation at low temperature, washed twice with pre-cooled PBS, then cultured with 1.0 MIC FSAL and the same volume of PBS (control group) suspension cells at 30 °C, and centrifuged at 0 h, 4 h and 8 h, respectively. Add 1 mL of Fluo-3 AM working liquid diluted to 5 μM with HBSS buffer, culture for 20 min at 37 °C in the dark, and add 5 mL of HBSS for 40 min. After culture, the cell cells were washed with HEPES buffer saline for three times and then suspended, and incubated at 37 °C in darkness for 10 min. After incubation, intracellular fluorescence intensity was detected by fluorescent enzyme marker, and observed by fluorescence microscope and photographed.

Determination of cytochrome c (cyt c)

The spore suspension of *R. nigricans* was prepared and the content of cytochrome c in *R. nigricans* was determined by ELISA kit.

Determination of hydrogen peroxide (H₂O₂) content

The spore suspension of *R. nigricans* was added into the PDB medium and cultured at 28 °C in a constant temperature rotating table at 135 r/min for 48 h. After filtration with four layers of cloth, the mycelium was weighed with 1 g (wet weight) and added with FSAL to reach 1.80 mg/mL (1.0 MIC). The control group was added with the same amount

of sterile water. The treated mycelia were collected and the H_2O_2 content in *R. nigricans* was determined by H_2O_2 kit.

Determination of related enzyme activity

The enzyme activity of succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and ATPase activities assay were determined in accordance with the kit purchased from Nanjing Jiancheng Institute of Biological Engineering. The same weight of *R. nigricans* mycelia were treated as directed with FSAL (1.80 mg/mL) and pure water for 0, 4, and 8 h before the treated mycelia were collected for analysis of SDH, MDH, and ATP.

Determination of related genes expression

Four genes related to mitochondrial metabolism were selected: *TRINITY_DN3888_c0_g1*, *TRINITY_DN10135_c0_g2*, *TRINITY_DN13783_c0_g1*, *TRINITY_DN2752_c1_g1*. These genes encode succinate dehydrogenase (*SDHA*), voltage dependent anion channel (*VDAC*), NADPH oxidase (*NOX*), and adenine nucleotide transporter (*ANT*). Primers were designed from the Illumina sequencing data using premier 5 (Table 1). The actin gene sequence was used to create primers for an internal reference gene. Vazyme HiScript II Q RT SuperMix for qPCR Kit (VazymeBiotechCo., Ltd.) was used to create cDNA from extracted RNA. Then, using an ABI prism 7500 Fast qPCR, PCR reactions were carried out in the following ways: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, and finally 60 °C for 30 s. The target genes' relative expression levels ranged from 2 CT modalities. The relative expression levels of the target genes ranged from $2^{-\Delta\Delta CT}$ modalities (Zhao et al. 2022).

Statistical analysis

The SPSS program (Version 25.0, SPSS Inc., Chicago, IL, USA) was used to do statistical analysis on the experimental data after each experiment was carried out three times in

parallel. Comparisons across groups were made using one-way analysis of variance (ANOVA) in order to get experimental data. The $P < 0.05$ level was used to assess significant differences using the Duncan's multiple range test.

Results

Influence of different concentrations of FSAL on the incidence of strawberry

The effect of different concentrations of FSAL on the incidence of strawberry fruits is shown in Fig. 1. FSAL inhibited the growth of *R. nigricans* on strawberry fruits and showed a dose relationship, with increasing FSAL concentration, the incidence of fruits decreased and the inhibitory effect was significant (Fig. 1A). From Fig. 1B, it can be seen that at the 30 h of accessing *R. nigricans*, the incidence rate of the control group was 100%, the incidence rate of the 0.45 mg/mL FSAL treated fruits was 86%, the incidence rate of the 0.9 mg/mL FSAL was 40%, and the incidence rate of 1.8 mg/mL FSAL was 29%, which were significantly lower than that of the control group ($P < 0.05$). It is worth noting that the 0.9 and 1.8 mg/mL FSAL treatments were significantly lower than the control group ($P < 0.05$). There was no significant difference between the incidence rates of 0.9 and 1.8 mg/mL FSAL ($P > 0.05$).

MIC determination

The MIC measurement results of FSAL on *R. nigricans* are shown in Fig. 2. FSAL had a good inhibitory effect on *R. nigricans*. After two days of culture, there was no significant colony growth when FSAL concentration was ≥ 1.800 mg/mL, indicating that the MIC of FSAL inhibiting *R. nigricans* was 1.800 mg/mL. There was no significant difference from the result of the MIC of FSAL against *Botrytis cinerea* was 1.500 mg/mL (Wang et al. 2022).

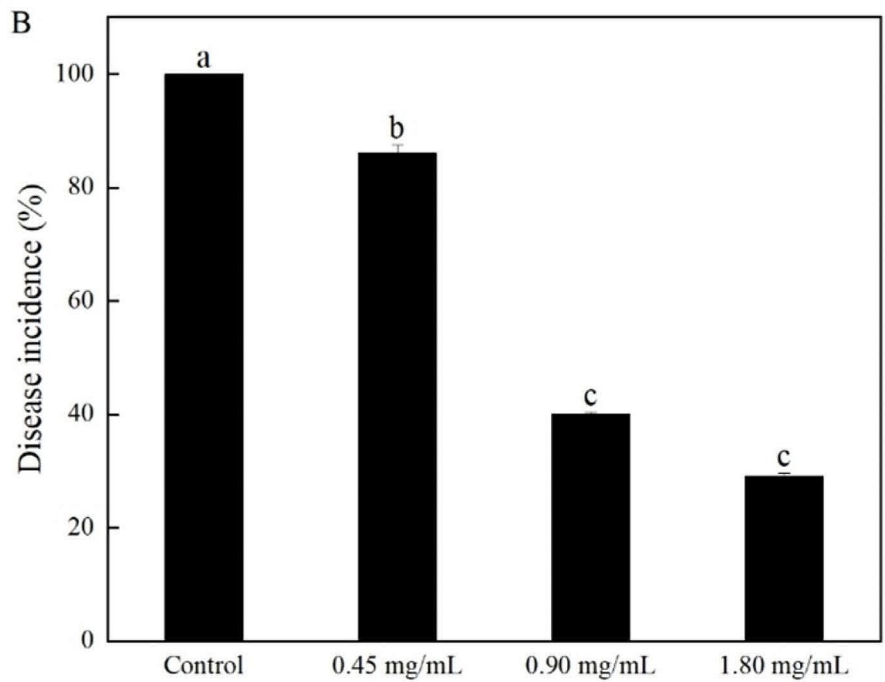
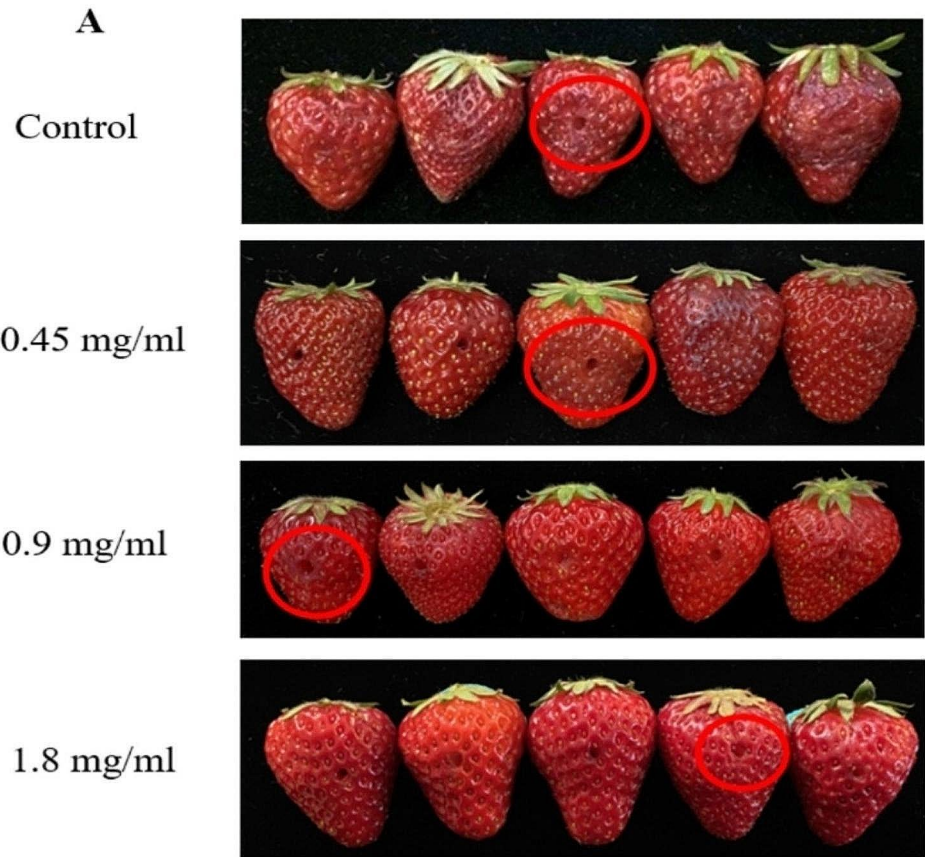
Influence of FSAL on apoptosis of *R. Nigricans*

As shown in Fig. 3, compared with the control group, the number of green fluorescent cells in the FSAL treatment group was higher at 4 h, with time effects, the number of stained spores increased with the extension of treatment time, and the induced membrane damage was more serious, the number of red fluorescent cells increased and the intensity of red fluorescence increased at 8 h.

Table 1 Sequence of oligonucleotide primers used for amplification of key genes for mitochondrial in *Rhizopus nigricans*

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>SDHA</i>	TTCAATGCTGATGCGGGTGC	AGACGGATTT CGGCTGTGC
<i>VDAC1</i>	TCCCGAGTGACCCAGAGCA	CCAGGCGAG ATTGACAGCA
<i>NOX2</i>	CGGCCCAACTGGGATAACG	TTCGGTCCG CAGAGGAAA
<i>ANT</i>	TCGTATGATGATGCAGTCT	CACCACCAGT ACCTCTAAG
<i>18s</i>	GTAGTCATATGCTTGCTC	ATTCCCCGTT ACCCGTT

Fig. 1 Influence of different FSAL concentrations on soft rot in strawberry fruit. **A** Representative strawberry samples on the 30 h of storage. **B** Disease incidence. In the bar graph, different letters (a, b, c) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation



Different concentrations of flavonoids from *Sedum aizoon* L.

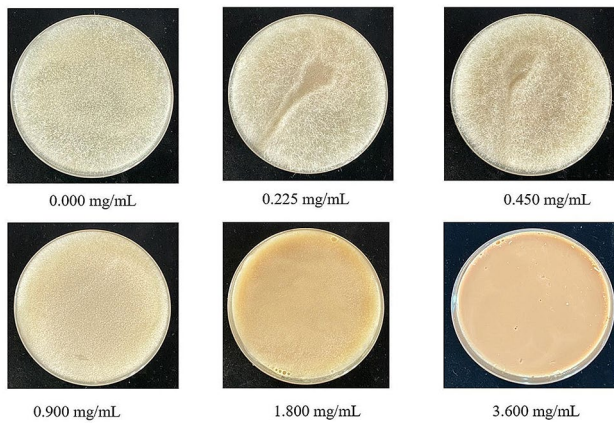


Fig. 2 The MIC of FSAL on *Rhizopus nigricans*. The pictures show the plate on the second day of incubation

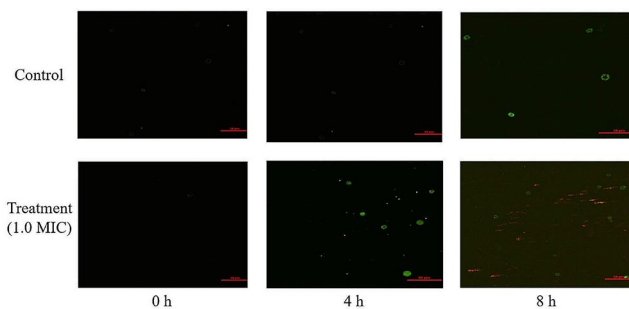


Fig. 3 Influence of FSAL on apoptosis of *Rhizopus nigricans*

Influence of FSAL on mitochondrial membrane potential (MMP)

After treatment with FSAL (1.0 MIC), the MMP of *R. nigricans* showed a decreasing trend. As shown in Fig. 4A, the regions on the Q 2 cell map represent the proportion of cells with MMP. With the increase of FSAL concentration, the fluorescence signal decreased from 93.4 to 44.1% and 36.4% in Q 2 region, while the change was small in the blank control group, from 89.2 to 84.3%. This indicated that FSAL treatment could reduce the MMP of *R. nigricans* in a time-dependent manner. The change of MMP was measured by measuring the fluorescence transformation of JC-1 from red (FL1) to green (FL2), as shown in Fig. 4B, FL2/FL1 showed a decreasing trend after FSAL treatment, and a significant difference ($P < 0.05$) appeared after 4 h treatment, with obvious time effect.

Influence of FSAL on mitochondrial permeability transition pore (mPTP)

As shown in Fig. 5A, at 0 h, there was no change in the permeability of *R. nigricans* mPTP between FSAL and control group. At 4 h and 8 h, compared with control group,

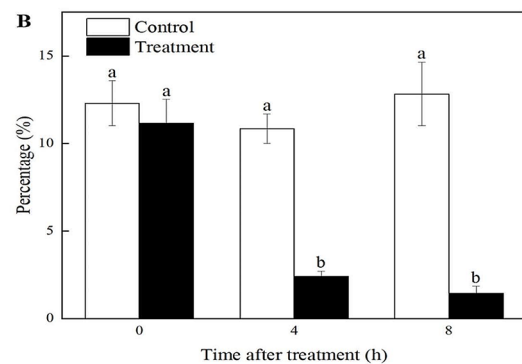
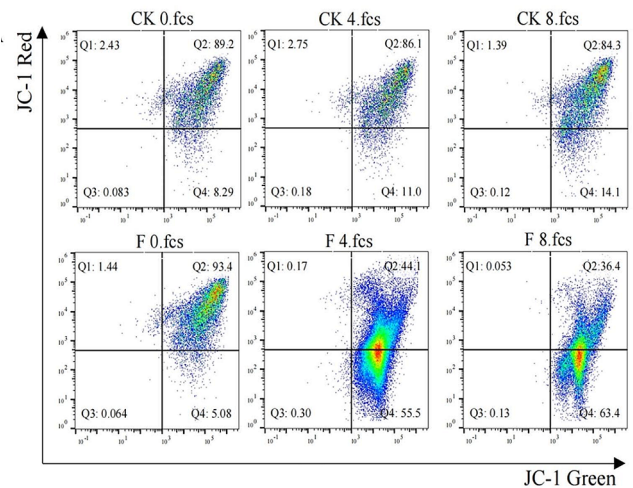


Fig. 4 Influence of FSAL on the mitochondrial membrane potential (MMP) of *Rhizopus nigricans*. **A** MMP flow cytometry analysis of *R. nigricans* treated by FSAL. **B** The ratio of normal cells to damaged cells. In the bar graph, different letters (a, b) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation

FSAL treatment significantly increased the openness of *R. nigricans* mPTP, thus weakening the green fluorescence of Calcein in mitochondria and reducing its fluorescence intensity. It shows a shift to the left in the peak plot. Accordingly, in Fig. 5B, the effect of FSAL on fluorescence intensity of MPTP showed a decreasing trend, and there was a difference at 4 h and 8 h ($P < 0.05$).

Influence of FSAL on Ca^{2+} content in *R. Nigricans*

Fluo-3/am is a fluorescent probe for the detection of intracellular calcium ions. When it exists in the form of free ligand, it does not emit any fluorescence, but when it enters the cell and binds to calcium ions, it can emit a strong green fluorescence. Under the action of external stimuli, the endoplasmic reticulum releases calcium ions, and then the mitochondria take up calcium ions. Mitochondrial Ca^{2+} overload leads to mitochondrial damage, *Cyt c* is released, caspase is activated, and apoptosis is induced. The increased calcium level is involved in the early signal transduction of apoptosis and the execution stage of apoptosis. Figure 6A

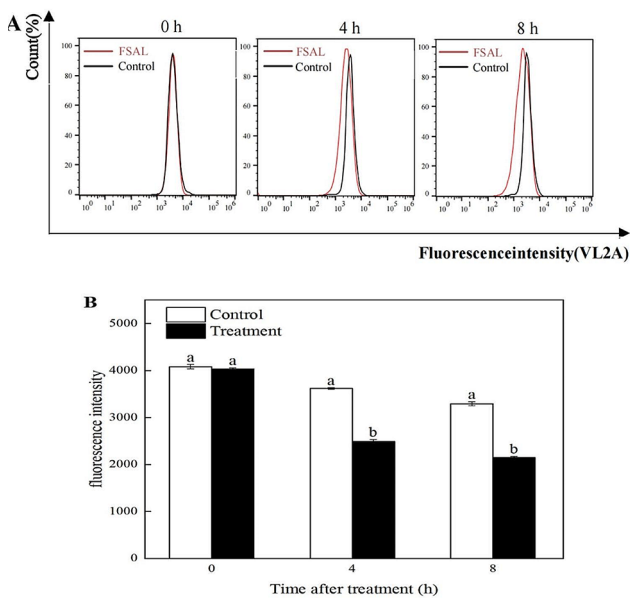


Fig. 5 Influence of FSAL on the mitochondrial permeability transition pore (MPTP) of *Rhizopus nigricans*. **A** MPTP of *R. nigricans* was detected by flow cytometry. **B** Effect of FSAL on fluorescence intensity of MPTP. In the bar graph, different letters (a, b) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation

and B display the fluorescence results obtained using a fluorescence microscope as well as data on the measured fluorescence intensity. As noted, the fluorescence intensity of the control group was lower and substantially different from that of the FSAL treatment group after 4 h ($P < 0.05$).

Influence of FSAL on cytochrome c (cyt c) of *R. Nigricans*

Results as shown in Fig. 7, the content of cytochrome c in mitochondria of *R. nigricans* was significantly reduced after treatment with FSAL, and showed a general downward trend with the extension of treatment time, and decreased to 8.75 ± 0.11 nmol/L after 4 h of treatment, which was significantly different from the control group. The lowest value was 8.53 ± 0.05 nmol/L at 8 h, and the content of Cyt c in mitochondria of the control group was 9.99 ± 0.21 nmol/L.

Influence of FSAL on hydrogen peroxide (H_2O_2) content in *R. Nigricans*

The content of H_2O_2 in mycelia (Fig. 8) was significantly increased after treatment with FSAL, reaching 2362.89 ± 109.32 mmol/g at 4 h treatment, which was significantly higher than that of the control group (709.90 ± 14.15 mmol/g) ($P < 0.05$). Subsequently, the content of H_2O_2 was basically constant, indicating that the accumulation of H_2O_2

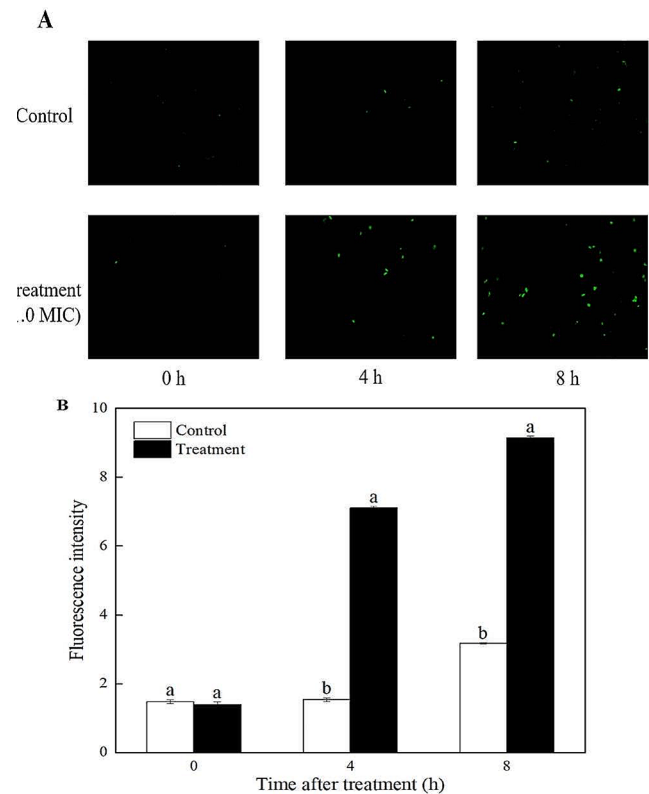


Fig. 6 Influence of FSAL on Ca^{2+} of *Rhizopus nigricans*. **A** Effect of FSAL on the accumulation of Ca^{2+} in *Rhizopus nigricans*. **B** Effect of FSAL on fluorescence intensity of Ca^{2+} . Various letters (a, b) show significant differences at $p < 0.05$ level. The error bar shows the standard deviation

was excessive, and *R. nigricans* was subjected to oxidative stress.

Influence of FSAL on related enzyme activity

As can be seen from Fig. 9A, the MDH enzyme activity of the flavonoid group showed a downward trend, and the enzyme activity at 4 h was 0.77 ± 0.09 U, while the treatment at this time was 1.15 ± 0.03 U, which was far less than the control group. And there is an obvious time effect.

As shown in Fig. 9B, the change of SDH activity in the control group was relatively gentle, while the SDH activity in the FSAL treatment group was generally declining. The SDH activity after 4 h treatment with FSAL was significantly lower than that in the corresponding control group. With the extension of treatment time, the SDH activity reached the lowest level at 8 h. These results indicated that FSAL treatment inhibited the activity of *R. nigricans* SDH.

As shown in Fig. 9C and D, the activities of $Na^+ K^+ -ATPase$ and $Ca^{2+} Mg^{2+} -ATPase$ in FSAL treated group were significantly decreased ($P < 0.05$), indicating that the ATPase activity of *R. nigricans* cells was affected after FSAL treatment. It is speculated that mitochondria were

Fig. 7 Influence of FSAL on cytochrome *c* (Cyt *c*) of *Rhizopus nigricans*. In the bar graph, different letters (a, b) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation

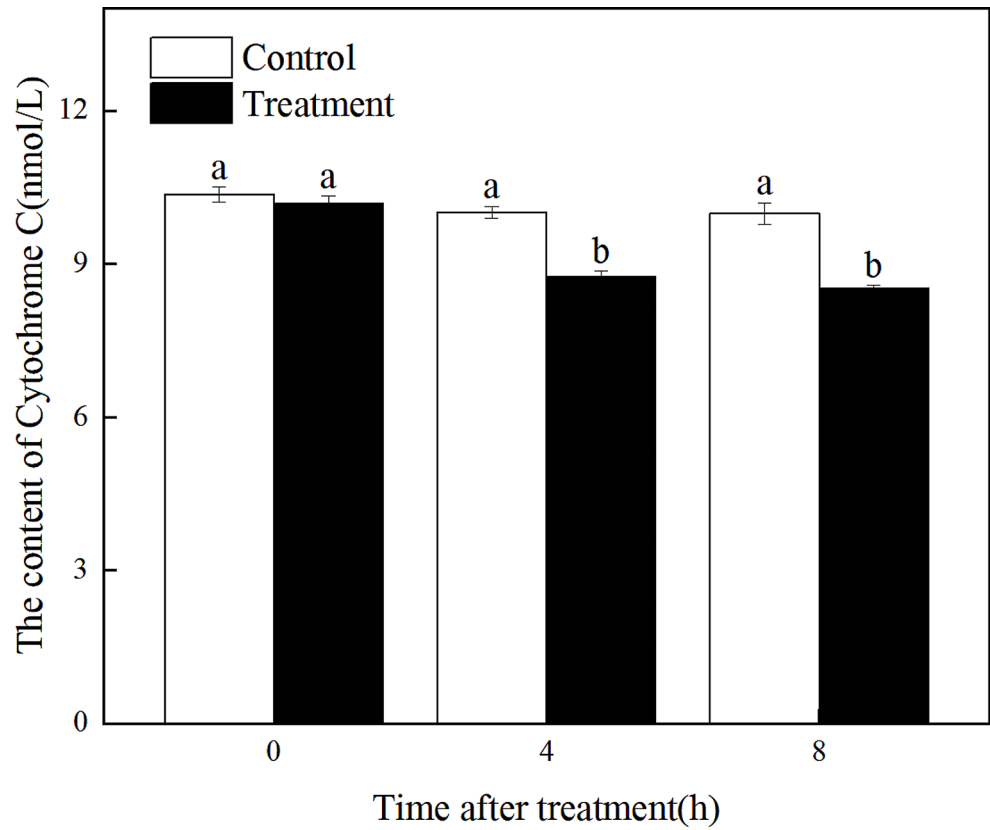
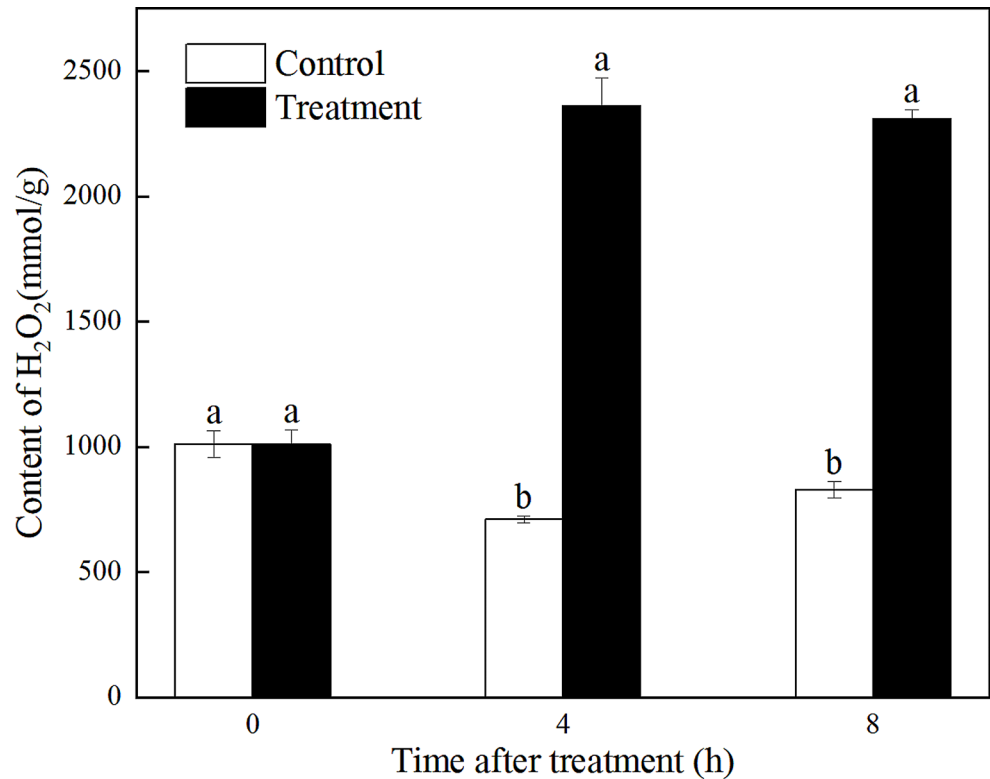


Fig. 8 Influence of FSAL on hydrogen peroxide (H_2O_2) content in *R. nigricans*. In the bar graph, different letters (a, b) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation



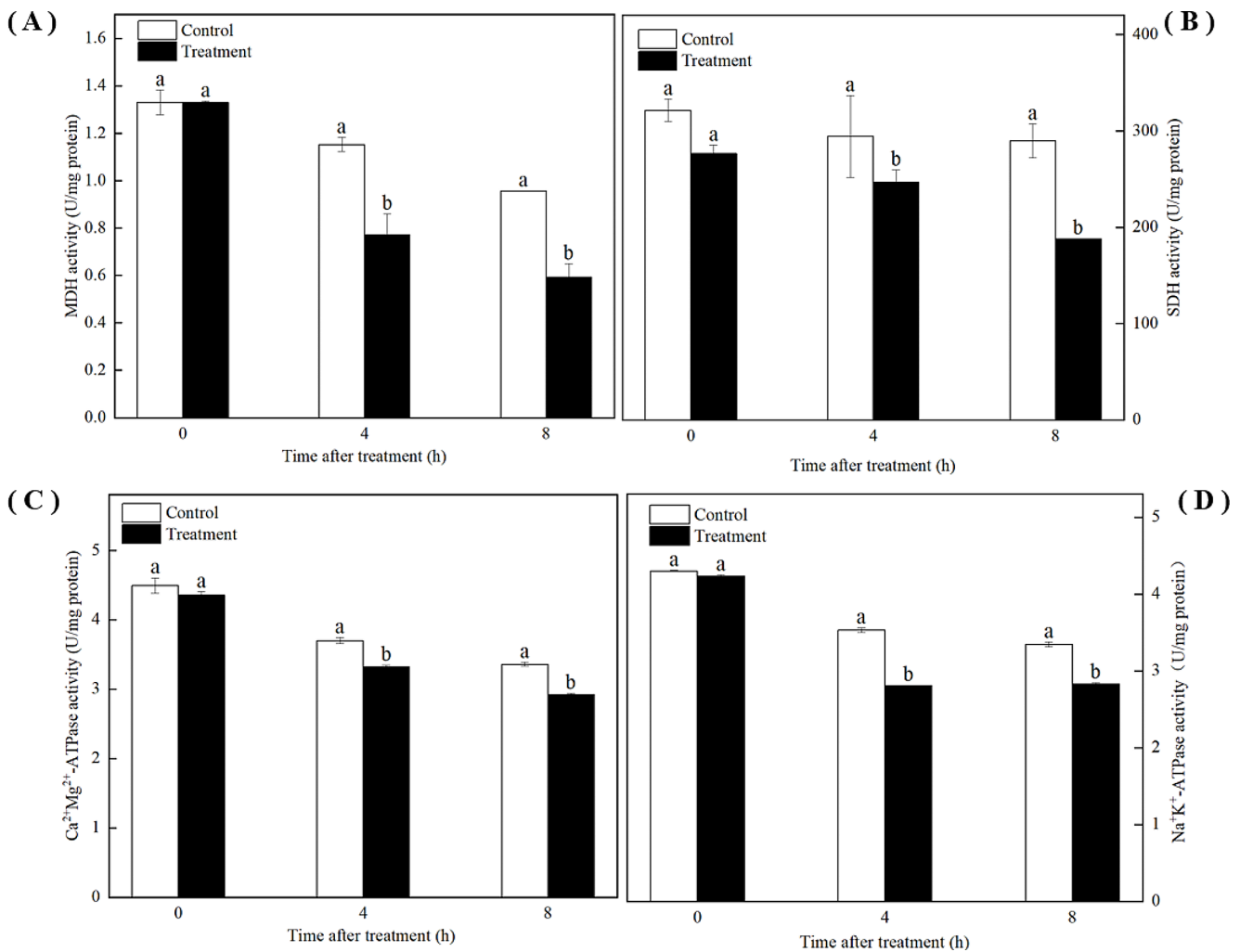


Fig. 9 Influence of FSAL on SDH, MDH and ATP activities of *Rhizopus nigricans*. **A** Effect of FSAL on SDH activity. **B** Effect of FSAL on MDH activity. **C** Effect of FSAL on Na⁺ K⁺-ATPase activity. **D** Effect

of FSAL on Ca²⁺ Mg²⁺-ATPase activity. In the bar graph, different letters (a, b) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation

damaged after flavonoid treatment, and energy metabolism was inhibited to a certain extent, thus affecting the energy supply of *R. nigricans* cells.

Influence of FSAL on the expression of related genes in mitochondrial

As shown in Fig. 10, mitochondria-related genes of *R. nigricans* were selected and their relative expression levels were analyzed to explore the influence of flavonoid on the fungi.

As shown in Fig. 10A, FSAL treatment significantly down-regulates *SDHA*, indicating that FSAL has an inhibitory effect on *R. nigricans*. In Fig. 10B, *VDAC1* was up-regulated in the FSAL treatment group, showing a significant difference from the control group ($P < 0.05$). From Fig. 10C, FSAL down-regulates *NOX2*, resulting in accumulation of ROS and damage of mitochondria. As shown in Fig. 10D, FSAL up-regulates *ANT1*, so it can be concluded that FSAL

treatment will cause the opening of *R. nigricans* mPTP, resulting in the occurrence of apoptosis in *R. nigricans*.

Discussion

Zou et al. (2023). demonstrated that the antimicrobial effect of 2-phenylethanol on strawberry fruits could reduce the natural decay of strawberries stored at ambient temperature and prevent the deterioration of strawberry organoleptic quality. Our findings similar with these previous reports. This result suggests that FSAL preserves freshness by inhibiting the growth of *R. nigricans* on strawberry fruits.

Phosphatidylserine (PS) externalization is a sign of early apoptosis of fungal cells. Under normal physiological conditions, phosphatidylserine is located on the inner side of the cell membrane. In the early stage of apoptosis, due to the lack of intracellular ATP, the concentration of cytoplasmic

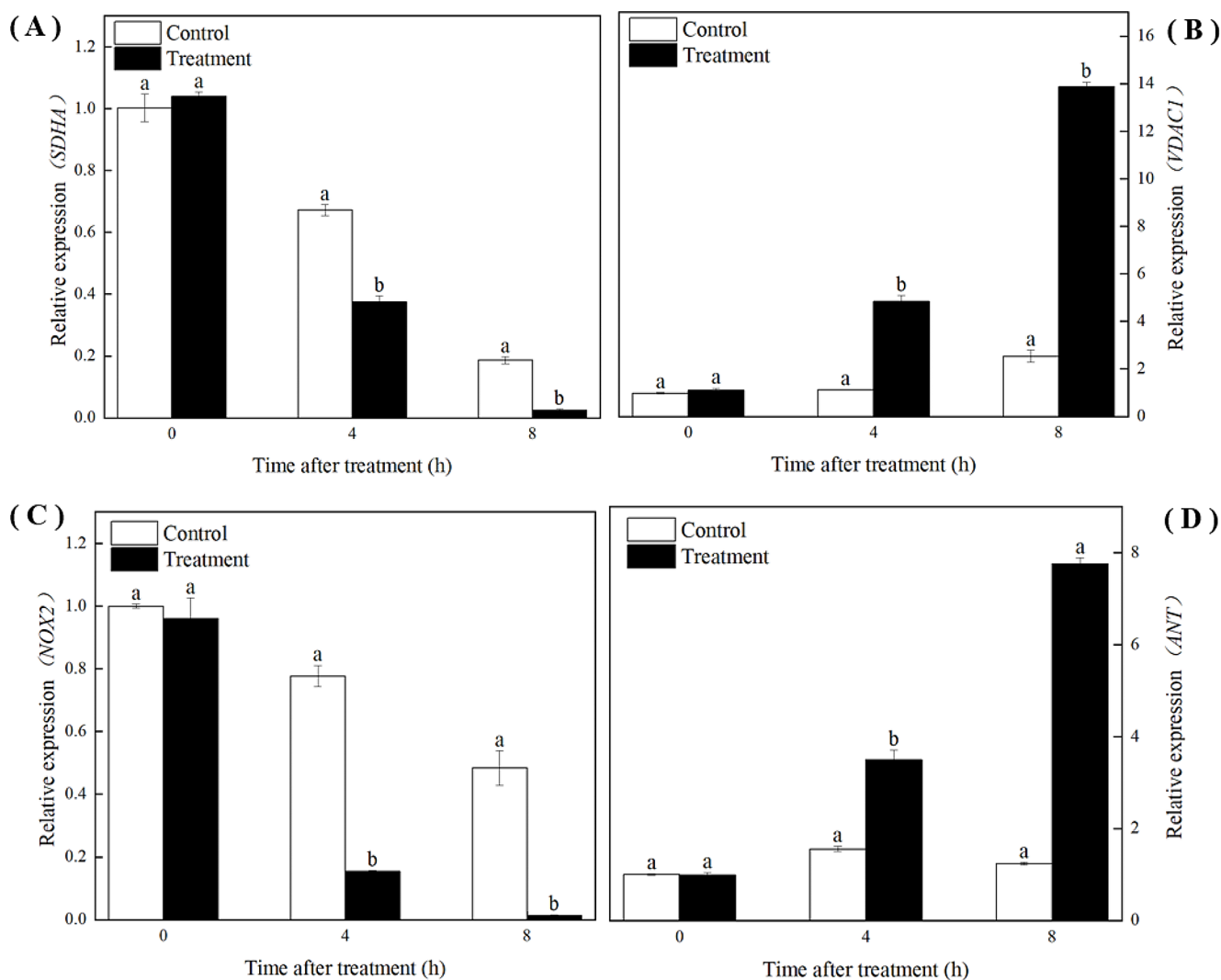


Fig. 10 The expression of major genes in mitochondrial metabolism of *Rhizopus nigricans*. **A** The expression of *SDHA*. **B** The expression of *VDACL1*. **C** The expression of *NOX2*. **D** The expression of *ANT*. In

the bar graph, different letters (a, b) indicate significant differences at $p < 0.05$ level. The error bar shows the standard deviation

Ca^{2+} increases, allowing it to flip to the membrane surface and bind specifically with Annexin V. Therefore, Annexin V can be used as an indicator for the detection of early apoptosis. Propidium Iodide (PI) is a red fluorescent dye that cannot penetrate cell membranes and can stain late apoptotic and necrotic cells. Annexin V in combination with PI can effectively distinguish early and late apoptotic cells. This suggests that mitochondrial function impairment is an important inhibitory mechanism of FSAL. These results (Fig. 3) showed that phosphatidylserine was turned from the inside of the cell membrane to the surface of the cell membrane, the number of late apoptotic cells was significantly increased by FSAL treatment, led to apoptosis. The results indicated that FSAL could induce the apoptosis of *R. nigricans*. Similarly, Shikonin concentration-dependent induced *Candida albicans* cell death, and the percentage of

apoptosis and necrosis increased significantly (Pang et al. 2023).

MMP is a necessary prerequisite for mitochondrial function, and the decrease of MMP caused by increased mitochondrial membrane permeability in fungal cells is one of the typical signs of early apoptosis (Fang et al. 2019). The JC-1 fluorescent probe aggregates in the mitochondrial matrix and excites red fluorescence when MMP is normal. In contrast, when depolarization occurs, MMP decreases and the JC-1 fluorescent probe is released from the mitochondria and reversed into a monomer that excites green fluorescence. These study decreases with the extension of FSAL treatment time (Fig. 4). 2-Chloro-1,3-dimethoxy-5-methylbenzo, which is highly lipophilic and of low molecular weight, inhibits the growth of *Candida albicans* by destroying the membrane of *Candida albicans*, reduces

MMP, leads to mitochondrial dysfunction, and thus induces the apoptosis of *Candida albicans* cells (Zhang et al. 2022).

The mPTP is the structural basis of mitochondrial permeability transition function and a protein-like channel at the junction of inner and outer membrane of mitochondria. During apoptosis or necrosis, mitochondrial contents are released into the cytoplasm through membrane channel pores (Frank 2019). The continuous opening of membrane channel pores results in the release of cytochrome c and the disappearance of mitochondrial membrane potential (Mao et al. 2018). Therefore, it is speculated that FSAL treatment increases the openness of mPTP, which may lead to apoptosis of *R. nigricans* (Fig. 5).

Mitochondrial matrix Ca^{2+} is a key regulator of ATP production and cell death. Ca^{2+} is the second messenger of many kinds of death signal transduction, and the increase of its level can cause mitochondrial damage, affect membrane permeability, and may lead to apoptosis (Zheng et al. 2020). In this study, the fluorescence intensity increased and the quantity of spores continuously increased throughout the course of the longer FSAL treatment period (Fig. 6). The result was dose-dependent. This result suggests that FSAL treatment contributes to the substantial accumulation of Ca^{2+} in *R. nigricans*, and then induce cell apoptosis.

After the release of cytochrome c from mitochondria, not only the mitochondrial respiratory chain and electron transfer are blocked, and the cell energy supply is reduced, but also after *Cyt c* enters the cytoplasm, it can hydrolyze caspase-9 into caspase-9 and further hydrolyze caspase-3. After caspase-3 is activated, cells enter an irreversible process of death. In addition, the study confirmed that *Cyt c* also has antioxidant function in mitochondria. Therefore, it is speculated that the release of *cyt c* into the cytoplasm will inevitably lead to an increase in the level of cell free radicals, and then cause cell apoptosis (Kadenbach et al. 2004). The results showed that flavonoid induced the release of cytochrome c in mitochondria of *R. nigricans* cells, suggesting mitochondrial damage (Fig. 7). Treatment with natural food flavor (E)-2-hexenal (E)-2-hexenal can reduce the *Cyt c* level in mitochondria of *Aspergillus flavus*, thus causing damage to the fungi (Ma et al. 2022).

Oxidative stress is caused by H_2O_2 in many species. Aquaporins allow this biochemically reactive substance to pass across cell plasma membranes with ease. Due to its high oxidation, it causes tissue damage in multicellular organisms by altering the intracellular redox balance, generating new molecules, and altering the membrane potential (Lennicke et al. 2015). H_2O_2 also induces apoptosis since it has a variety of harmful effects on the majority of cell types. The content of H_2O_2 in mycelia (Fig. 8) was significantly increased after treatment with FSAL, indicated the *R. nigricans* was subjected to oxidative stress.

MDH enzymes and SDH enzymes are one of the key enzymes in the TCA cycle of eukaryotic cells and play an important role in cellular energy metabolism. Adenosine triphosphate (ATP) provides direct energy for the physiological activities of the organism and is a necessary energy substance to ensure the life activities of the organism. The change of its content in the organism can directly indicate the intensity of the physiological activities of the organism (Maria et al. 2016). As we can see in Fig. 9, FSAL inhibited the activity of MDH and SDH in the tricarboxylic acid cycle, and also inhibited the activity of ATPase. The result is related to Essential oil derived from turmeric can inhibit the activities of MDH and SDH in *Aspergillus flavus*, and then affect ATP synthesis and interfere with normal metabolic processes (Hu et al. 2017).

Succinate dehydrogenase (*SDH*) plays a role in regulating the growth and metabolism of microorganisms in the tricarboxylic acid cycle (TCA). Voltage-dependent anion channels (*VDAC*) are also known as mitochondrial porins (Schein et al. 1976), plays a key role in mitochondria-mediated apoptosis. Its function is to control the transport of metabolites into and out of mitochondria and the production of energy (Shoshan-Barmatz et al. 2010), release apoptotic proteins located in the intermembrane space, and is related to pro-apoptotic proteins and anti-apoptotic proteins. Therefore, *VDAC1* is considered to be a promising target for regulating apoptosis. Study have shown that inhibiting *VDAC1* can significantly reduce the occurrence of apoptosis caused by the abnormal opening of mPTP (Liu et al. 2016). NADPH oxidase (*NOX*) participated in the removal of ROS. When the activity of *NOX* is inhibited, ROS accumulation is caused, and excessive ROS will also lead to lipid peroxidation of mitochondrial membrane. In recent years, several reports have suggested that mPTP is the main target of ROS in mitochondria (Marchi et al. 2012). The effect was found in thymol and salicylic acid treated *Rhizopus stolonifer*, leading to accumulation of ROS and impairment of mitochondria-related functions (Kong et al., 2019). The mitochondrial transport family member adenine nucleotide translocator (*ANT*) is crucial for intracellular energy metabolism. Epstein–Barr virus can promote the abnormal opening of mPTP in host cells by up-regulating the expression of *ANT1*, and the increase of *ANT1* expression is an important indicator to detect the abnormal opening of mPTP (Zhao et al. 2021).

In conclusion, we found that mitochondria obtained from *R. nigricans* extracts were more sensitive to FSA. FSAL hinders ATP production by affecting the *R. nigricans* TCA cycle and decreasing ATPase activity, thus affecting cellular energy supply. The intracellular Ca^{2+} content of *R. nigricans* under FSAL treatment was increased dramatically, and Ca^{2+} overload occurred phenomenon, and the determination

of mitochondrial permeability transition pore (mPTP) revealed that mPTP opening increased substantially and with the prolongation of FSAL treatment, and the opening of mPTP was irreversible, which resulted in mitochondrial swelling or even rupture once the mitochondrial permeability transition pore was opened. This suggests that FSAL may cause damage to mitochondrial function, which in turn causes the phenomenon of apoptosis. It was also found that FSAL decreased MMP, which led to the accumulation of H₂O₂ content and the decrease of Cyt *c* content, destroying the activity of mitochondria-related genes.

Acknowledgements This study was supported by Natural Science Foundation of Zhejiang Province [LY16C200003]. We thank Key Laboratory of Animal Protein Food Processing Technology of Zhejiang Province.

Author contributions QG: Methodology, Investigation, Writing - Original Draft. SZ: Investigation, Writing - Original Draft. XS: Writing - Review & Editing. YW: Data Curation. JC: Validation, Formal analysis. HW: Conceptualization, Supervision, Project administration. FX: Resources, Investigation.

Data availability Data will be made available on request.

Declarations

Competing interests The authors declare no competing interests.

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