Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(5):12-20

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

The possible mechanism of antifungal activity of cinnamon oil against *Rhizopus nigricans*

Yaru Li[1], Ying Nie[1], Linyan Zhou[1], Shurong Li[2]*, Xuanming Tang[1], Yang Ding[1] and Shuying Li[1]

[1]Key Laboratory of Agro-product Processing and Quality Control/Institute of Agro-Products Processing Science and Technology, Chinese Academy of Agricultural Sciences, Beijing, China
[2]Beijing Vocational College of Agriculture, Beijing, China

ABSTRACT

Cinnamon oil has been confirmed as a useful antifungal agent to inhibit Rhizopus nigricans. However, the antifungal mechanism has not been clearly revealed. The possible mechanism as follows were investigated, such as the effects of cinnamon oil on the cell morphology, cell membrane and the activities of the key enzymes in tricarboxylic acid (TCA) cycle. Cinnamon oil can inhibit the mycelia growth of R. nigricans, and scanning electron microscope (SEM) observations revealed that the mycelia morphology alterations of R. nigricans were the markedly shriveled and collapsed hypha, even flatted empty hyphae, swelled cell wall, disrupted plasma membrane, with cytoplasmic matrix leakage. Furthermore, cinnamon oil inhibited the biosynthesis of ergosterol significantly, damaging the cell membrane structure, causing the leakage of intracellular ions, protein and the higher absorbance at 260nm. Moreover, cinnamon oil affected the energy metabolism of R. nigricans by decreasing the activities of succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) in tricarboxylic acid (TCA) cycle.

Key words: cinnamon oil, *Rhizopus nigricans*, antifungal mechanism

---

INTRODUCTION

Postharvest losses in harvested fruit and vegetables reach very high values, representing more than 50% of the total production in developing countries and more than 25% in industrialized countries, if we did not adopt optimal postharvest handling and storage technology [1]. Much of these losses are owing to the attack of fungal pathogens because of the high amount of water content and nutrients of fruits. The pathogens that cause the most postharvest diseases are belonging to *Aspergillus*, *Alternaria*, *Botrytis*, *Mucor*, *Colletotrichum*, *Rhizopus*, *Penicillium* and other genera [1] *R. nigricans* is a major cause of plum, peach, apricot and other stone fruits postharvest diseases [2]. People always apply the synthetic fungicides to control the diseases, however, the chemical fungicides have toxicological and environmental risks. Many researchers have focused on the study of natural fungicides such as essential oils in recent years.

Cinnamon oil is one of essential oils as an aromatic oily liquids obtained from twigs, bark, leaves, et al of *Cinnamomum cassia* that belongs to *Lauraceae* family and usually grows in South and South-East Asia [3]. Previous studies have shown that cinnamon oil is useful as a food preservative to inhibit the growth of food-related microorganisms, such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Rhizopus species*, *Aspergillus* species and *Penicillium* species et al [4-7]. Cinnamon has been used in medicine and granted generally recognized as safe (GRAS) status as a food additive by the FDA [7]. The main compounds of cinnamon oil trans-cinnamicaldehyde, cinnmyl cinnamate, andbenzyl cinnamate et al which are responsible for the antimicrobial activity [8]. In our previous study, cinnamon oil demonstrated significant antifungal activity against *Rhizopus nigricans*, and the minimum inhibitory concentration (MIC) was 0.1μL/mL. However, the mechanism of the action

against fungi has not been identified clearly. The aims of this research were (1) to study the antifungal activity of cinnamon oil against *R. nigricans*, (2) to reveal the possible mechanism of cinnamon oil against *R. nigricans* including the hyphal morphology, the effects of cinnamon oil on cell membrane, the activities of SDH and MDH.

EXPERIMENTAL SECTION

1.1 Essential oil

The cinnamon oil was bought from Ecoarts Enterprise (Shanghai) Co., Ltd.

1.2 Fungi and culture

The *R. nigricans* was isolated from dried apricots. It was grown for 7 days on potato dextrose agar (PDA) at 28°C before the experiment. The fungal strain cultures were maintained on potato dextrose agar (PDA) at 4°C. The old cultures were transferred to a fresh medium every two months to avoid the decline of strain viability.

1.3 Experimental methods

1.3.1 Fungi incubation and sample collection

An aliquot of 1 mL *R. nigricans* suspension (10^7 spores/mL) was inoculated into 100 mL Potato dextrose broth (PDB) medium and incubated at 28°C, 160 rpm for 2 days, and then 1 MIC (0.1μL/ml, obtained from another experiment), 0.5 MIC (0.05μL/mL) cinnamon oil were added in the fungal suspension respectively, no addition was into the control group. The suspensions were incubated at 28°C, 160 rpm for 24 h. Samples from the suspensions were collected at 3 h intervals during the incubation. The suspensions collected at different time were centrifuged at 5000 rpm for 10 min to obtain mycelium and supernatant, and then the mycelium was washed with distilled water twice. Each test was run in triplicate.

1.3.2 Antifungal effects of cinnamon oil on the wet mycelia weight

The effect of essential oil on the wet mycelia weight was determined by the method of Tian et al [9]. Mycelium was collected as before, and then the weight of mycelium was determined by using electronic balance (YP10002, Shanghai Youke Instrument Co., Ltd. Shanghai, China).

1.3.3 SEM observations

To examine the effects of cinnamon oil on fungal cell morphology, SEM studies were carried out as reported with some modifications [9-10]. After incubation, mycelium exposed to cinnamon oil were fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH=7.4) for 2 h at room temperature. After fixation, the samples were dehydrated in a graded ethanol series (30, 50, 70, 80, 90% and two times at 100%) for a period of 10 min in each series. At last, they were dipped into pure isoamyl acetate replacement two times, each time for 30 min. The samples were dried by sublimation in a freeze dryer (ES-2030, Hitachi, Tokyo, Japan), then gold-coated using a sputter coating machine (IB-5, Eiko, Japan) for 60 s. These samples were examined in a SEM (QUANTA 200F, FEI, USA).

1.3.4 Effects of cinnamon oil on cell membrane

The suspensions collected at different time were centrifuged at 5000 rpm for 10 min to obtain supernatant. The electric conductivity, the amount of protein and the absorbance at 260 nm ($A_{260\ nm}$) of the supernatant were determined to explain the changes of membrane permeability and the release of the cellular material. According to Paul et al. [11] and Shao et al. [9], the electric conductivity and the absorbance at 260 nm of the obtained supernatant were measured in a conductivity meter (DDS-307, Shanghai Precision Scientific Instrument Co., Ltd. Shanghai, China) and an UV/Vis spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd., Beijing, China), respectively. The determination of the amount of protein was carried out according to the work of Bradford [12] with bovine serum albumin as a standard.

1.3.5 Determination of ergosterol content in the cell membrane

Cellular ergosterol was examined as described by Tian et al. [13] and Brilhante et al. [14], with some modifications. The ergosterol content was counted as a percentage of the wet weight. An aliquot of 10 mL 25% alcoholic potassium hydroxide solution (35 mL of sterile distilled water and 25 g of KOH, brought to 100 mL with 100% ethanol) was added into every sample and mixed for 2 min by vortex (SK-1, Shanghai Yiheng Instruments Co., Ltd., Shanghai, China), then incubated at 85°C for 4 h. After the incubation, samples were allowed to cool to room temperature. Ergosterol was extracted from each sample by adding n-heptane. 5 mL n-heptane was added into the mixture and mixed for 2 min by vortex (SK-1, Shanghai Yiheng Instruments Co., Ltd., Shanghai, China) and then stood for 1 h at room temperature to allow the layers to separate. The n-heptane layer was measured by an UV/Vis spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd., Beijing, China) at 282 nm and compared to a predetermined standard curve [14].

1.3.6 Determination of dehydrogenase activity

1.3.6.1 Effect of Cinnamon oil on SDH of *R. nigricans*

The activity of SDH was examined as described by Yao et al. [15], with some modifications. Each approximately 2 g wet mycelium was re-suspended in 10 mL phosphate buffer (PBS, pH 7.4), and then broken with ultrasound in ice bath for 10 min (550 W, working for 3 at 3 s intervals), then centrifuged at 5000 rpm for 5 min at 4°C in order to remove mycelia fragments and conidia. The supernatant was assayed using the SDH assay kit purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China) depending on the statements of the manufacturer. The activity of SDH can be expressed:

1 U/mg prot=ΔA_{600}/(0.01·min·mg protein)

1.3.6.2 Effect of Cinnamon oil on MDH of *R. nigricans*

The activity of MDH was examined by some modifications of the assay described by Yao et al. [15]. Each approximately 2 g wet mycelium was re-suspended in 10 mL phosphate buffer (PBS, pH 7.4). Cell suspensions were broken with ultrasound in ice bath for 10 min (550 W, working for 3 at 3 s intervals), and then centrifuged at 10000 rpm for 30 min at 4°C. The supernatants were collected and stored at ice temperature for MDH measurements. The activity of MDH was assayed by using the MDH assay kit purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China) according to the statements of the manufacturer. The activity of MDH was expressed as:

1 U/mg prot=ΔA_{340}/ (0.01·min·mg protein)

1.4 Statistical analysis

All experiments were performed in triplicate. A statistical package (SPSS, version 17.0 for Windows, SPSS Inc.) was used for the data processing. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

2.1 Antifungal activities of Cinnamon oil on the wet mycelia weight

Cinnamon oil and its main compounds are known to exhibit broad antimicrobial activity. The effects of cinnamon oil on the mycelia growth of *R. nigricans* are shown in Fig.1. Cinnamon oil inhibited the growth of *R. nigricans* with a dose-dependent manner. The wet mycelia weight of control group increased 1.421 g and no significant ($P \leq 0.05$) mycelia growth was recorded with cinnamon oil treatment. Cinnamon oil at 1 MIC caused the higher inhibition of mycelia growth than 0.5 MIC. The results indicated that cinnamon oil exhibited antifungal activity on the mycelia growth of *R. nigricans*, and the mycelia growth was recorded to change with increasing oil concentrations and incubation time. Xing et al. [7] found that the MIC of cinnamon oil against *R. nigricans* was 0.64% (v/v), however, our studies showed that no mycelia growth was recorded at 0.1 μL/mL. This difference occurred may be attributed to the different components of these two kinds of cinnamon oils.

2.2 Effects of cinnamon oil on cell morphology

The changes in the cell morphology of *R. nigricans* are presented in Fig.2. Untreated cells showed an appearance of smooth surface and oval shape with uniform and robust hyphae of constant diameter in 24 h (Fig.2a–e). The surface of cells treated with cinnamon oil at 0.5 MIC began to shrink at 6 h (Fig.2g). The hyphal structure underwent greater morphological changes with clear foam, dissolved cell walls and leakage of cytoplasmic matrix at 12 h (Fig.2h). Cells began to repair and grow after 18 h (Fig.2i) and the same with the control at 24h (Fig.2j). Cells treated with cinnamon oil at 1 MIC were damaged more significantly than 0.5 MIC, accompanying with collapsed and flattened empty hyphae, and died ultimately (Fig.2k-o). The damage was more serious with the extension of time periods. Shao et al. [9] and Tian et al. [10] obtained similar results, and they found that essential oil could result in degenerately modifications of fungal microstructure, such as decreased hyphal diameters, vacuolated, markedly shriveled and crinkled hypha, collapsed and flattened empty hyphae, plasma membrane disruption and swelling of hyphal wall.

2.3 Effects of cinnamon oil on cell membrane

The electric conductivity (Fig.3) in the PDB culture media all significantly ($P \leq 0.05$) increased with the incubation time after cinnamon oil treatment. The $A_{260\ nm}$ (Fig.4) of culture media treated with cinnamon oil increased sharply after the treatment for 3 h, and then the 0.5 MIC group got to the balance, the 1 MIC group got to the balance after 6 h. However, the mount of protein (Fig.5) in the PDB culture media increased in 12 h, then began to decrease at the concentration of 0.5 MIC. Meanwhile it continued to increase in incubation time at the concentration of 1 MIC. The result indicated that the fungal cells could repair themselves after 12 h with the antibacterial activity of cinnamon oil reducing at 0.5 MIC. All the samples treated with cinnamon oil at 1 MIC showed higher values of these parameters than those of 0.5 MIC.

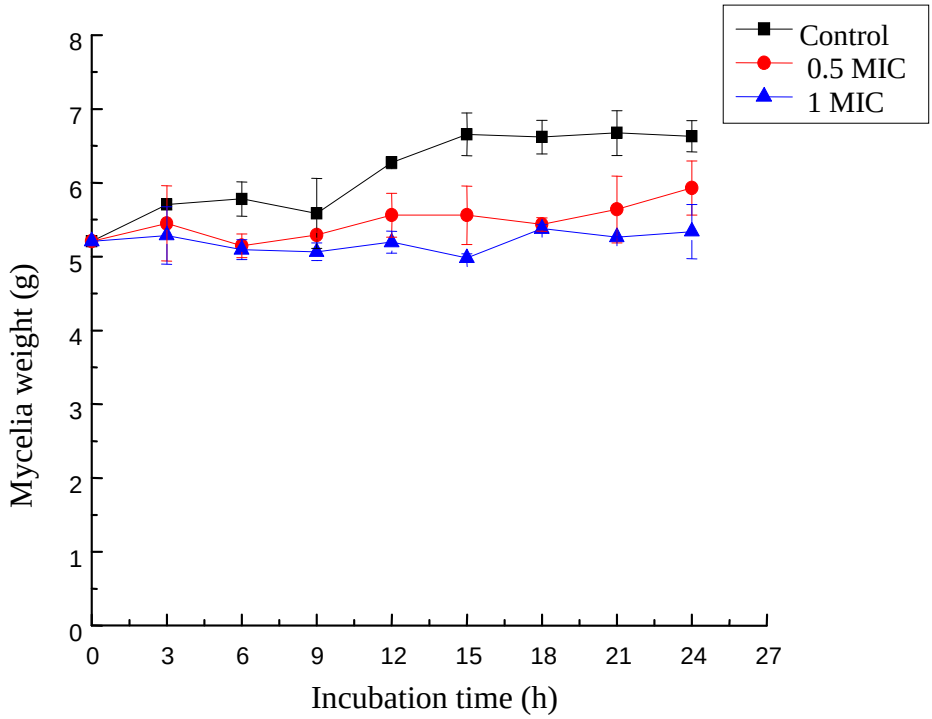

Fig.1: Effect of Cinnamon oil on mycelia weight of *Rhizopus nigricans*

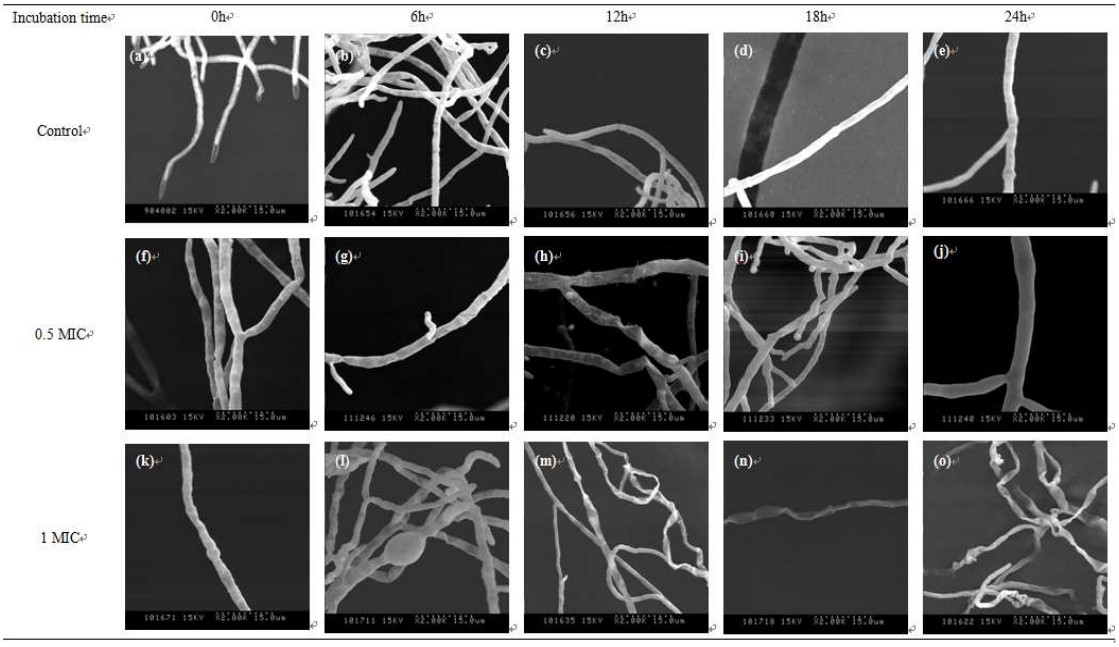

Fig.2: Scanning electron microscopy（SEM）of *Rhizopus nigricans* treated with Cinnamon oil over time

The microbial cell membrane plays an important role in maintaining cell normal life. It isolates the intracellular material from environment and maintains the material and energy balance. It is generally said that the cell membrane which is the target for antibacterial agents [16]. Burt [4] proposed that the hydrophobic compounds of essential oil are responsible for their partition into the lipid layer of the cell membrane, resulting in a change in permeability and a consequent leakage of cell constituents. Membrane damage can be demonstrated by measuring the leakage of intracellular contents, such as reducing sugar, K+, protein, ATP, RNA, nucleotides, et al. [16]. In our study, a significant loss of cell constituents has been observed with cinnamon oil treatments. Other antimicrobial compounds, such as mustard essential oil [17], phenyl aldehyde and phenyl propanoids[18], oregano-basil EO combination[8], *Melaleuca alternifolia* oil (Tea Tree Oil, TTO) [19] also induced the release of cell constituents increasing. Shao et al.

[10] evaluated the inhibitory effects of TTO against *Botrytis cinerea* in vitro. The antifungal mechanism of TTO was associated with its effects on the permeability of cell membrane, performing the increasing electric conductivity and the absorbance at 260 nm. Sun et al. [20] showed that rosemary acid resulted in the leakage of reducing sugar and protein of *Escherichia coli* and *Staphylococcus aureus.* All of these results suggest that the cytoplasmic membrane is damaged. Our study led to a similar conclusion that the membrane structure of the fungal is significantly impaired by cinnamon oil. The increased cell release, electric conduction and protein leakage were considered to be a direct result of the damaged membrane.

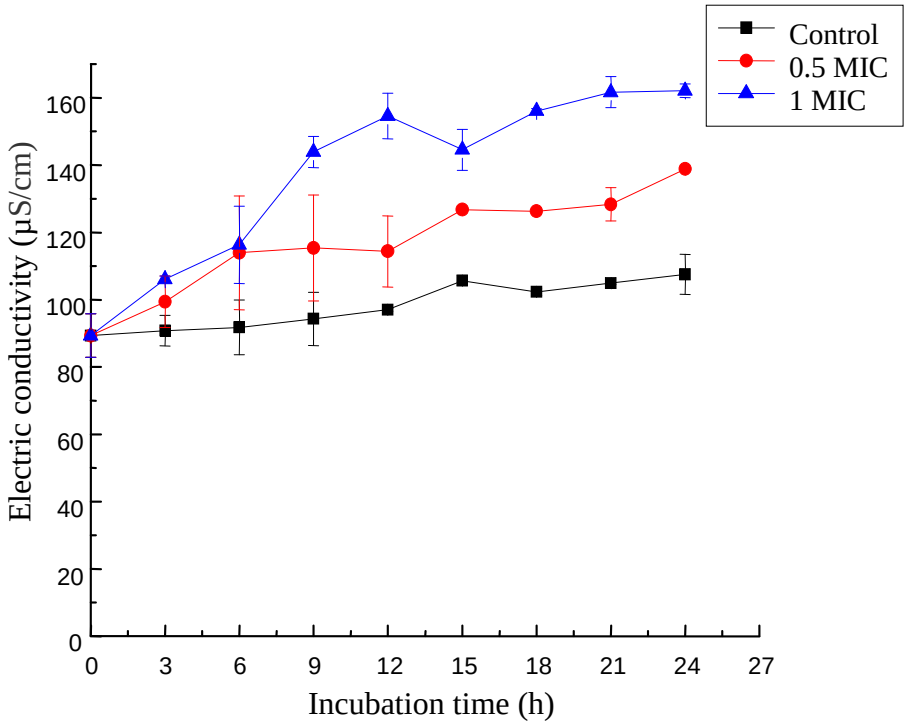

Fig.3: Effect of Cinnamon oil on electrical conductivity of *Rhizopus nigricans*

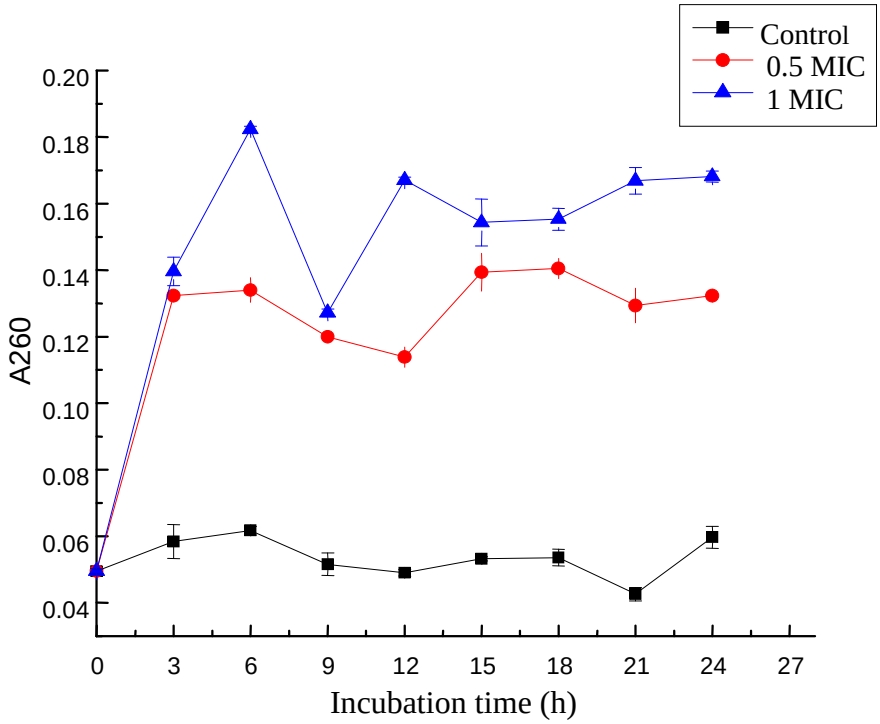

Fig.4: Effect of Cinnamon oil on cell release of *Rhizopus nigricans*

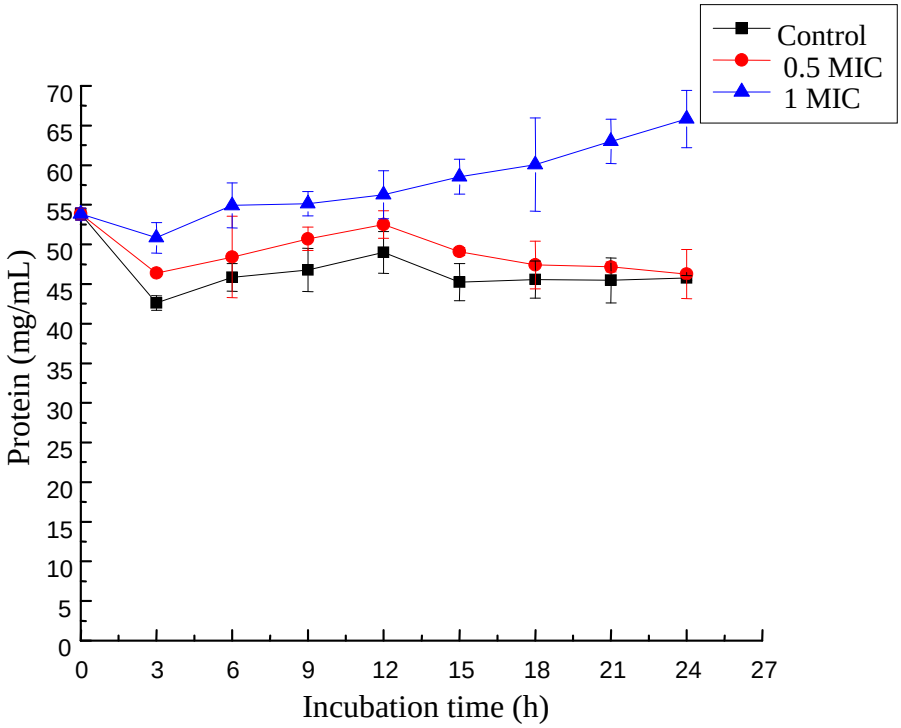

Fig.5: Effect of Cinnamon oil on protein of *Rhizopus nigricans*

2.4 Effects of ergosterol content in the plasma membrane

The effects of cinnamon oil on the ergosterol amount in the cell membrane of *R. nigricans* are shown in Fig.6. There was not significant (P≥0.05) change with the control samples. Meanwhile the ergosterol content first increased, and then began to decrease with the treatment of cinnamon oil at the concentration of 0.5 MIC. However it decreased continuously in incubation time period at the concentration of 1 MIC. And higher concentrations of cinnamon oil resulted in smaller amounts of ergosterol from the fungal cells. The results demonstrated that the ergosterol quantity (at 282 nm) in the plasma membrane of *R. nigricans* was significantly inhibited by cinnamon oil and showed a dose-dependent decrease.

Ergosterol is the major sterol constituent of the fungal plasma membrane, responsible for maintaining membrane fluidity, asymmetry, integrity and the cell functions [18]. Some studies have shown that series of compounds such as CJEO oil [21], phenyl acetaldehyde and eugenol [18], dill oil [13] et al. can target the ergosterol of membrane. These compounds can either inhibit the biosynthesis of ergosterol or bind with ergosterol to disrupt the membrane integrity compared to azole antifungal drugs, which is a kind of well known inhibitors of ergosterol biosynthesis. Khan et al. [18] had suggested that phenyl aldehyde and propanoids class of compounds could inhibit ergosterol biosynthesis and simultaneously bind with ergosterol, resulting in the membrane damaging. The main compound of cinnamon oil is cinnamicaldehyde [8] that has the same aldehyde groups, so we could get similar result that cinnamon oil can decrease the quantity of ergosterol with the increasing concentration by inhibiting the normal ergosterol biosynthesis.

2.5 Effects of dehydrogenase activity

The effects of cinnamon oil on the SDH and MDH activities in *R. nigricans* cells are presented in Fig.7 and Fig.8. The SDH and MDH activities of control group only showed a slight change within the incubation time period for 24 h, and the activities all decreased after cinnamon oil treatment. The SDH activity of 0.5 MIC group reached the minimum of 1.05 U/mg prot when incubated for 12 h, and then increased. The SDH activity of 1 MIC continued to decrease in incubation time, and it almost lost the activity at the end of incubation time. MDH values changed as similar as the regulation of SDH with a little light. The MDH value of 0.5 MIC group was 5.58 U/mg prot when incubated for 12 h. The MDH value was 1.33 U/mg prot at the end of incubation when treated with cinnamon oil at the concentration of 1 MIC. In general, the dehydrogenase activities of the treated group were lower than the control group throughout the examined incubation period. The change in the activities of SDH and MDH of fungal cell treated with cinnamon oil followed the same pattern as that.

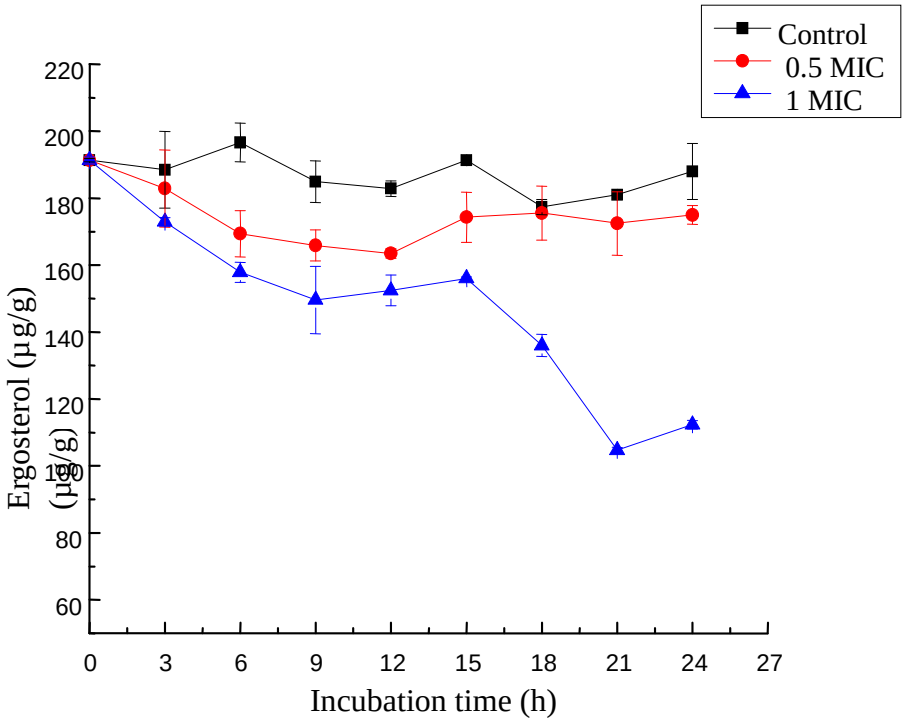

Fig.6: Effect of Cinnamon oil on ergosterol of *Rhizopus nigricans*

Succinate dehydrogenase plays an important role in the cellular energy metabolism of microbes, and its activity reflects the energy metabolic status of the bacterial cell. Malate dehydrogenase, enzyme of NADP+, forms NADPH by accepting hydrogen from metabolites during biosynthesis [15]. Our studies showed that cinnamon oil has pronounced inhibitory effect on the dehydrogenase activities. Similarly, terpenes and protamine could significantly inhibit the activities of both SDH and MDH [22]. Yao et al. [15] suggested that nobiletin and tangeretin changed the enzymes structures by reacting with their side chain groups, resulting in conformational changes of the enzymes which decreased enzyme activity.

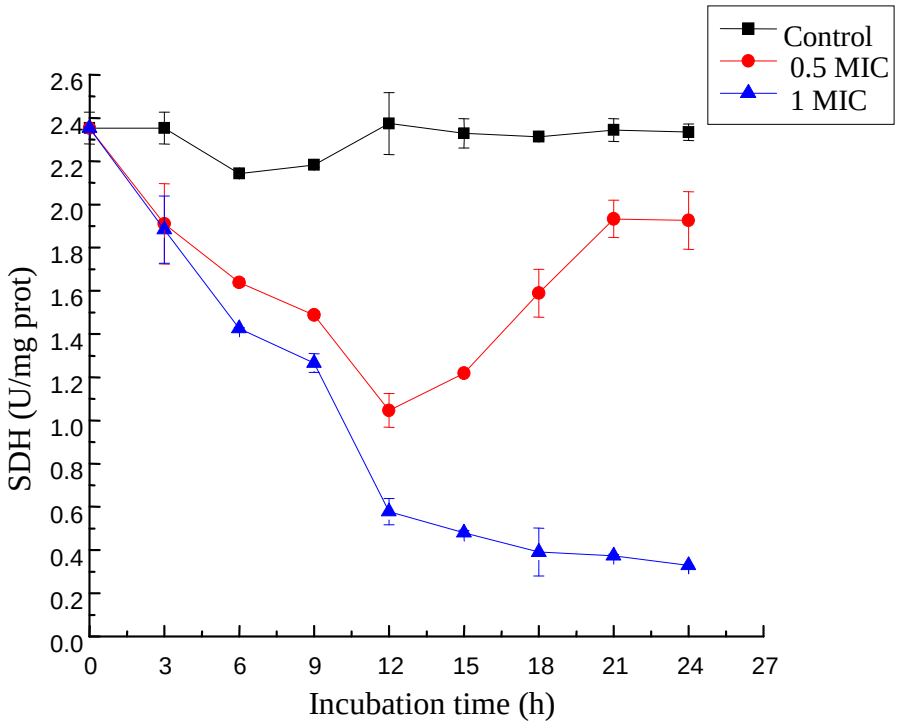

Fig.7: Effect of Cinnamon oil on SDH of *Rhizopus nigricans*

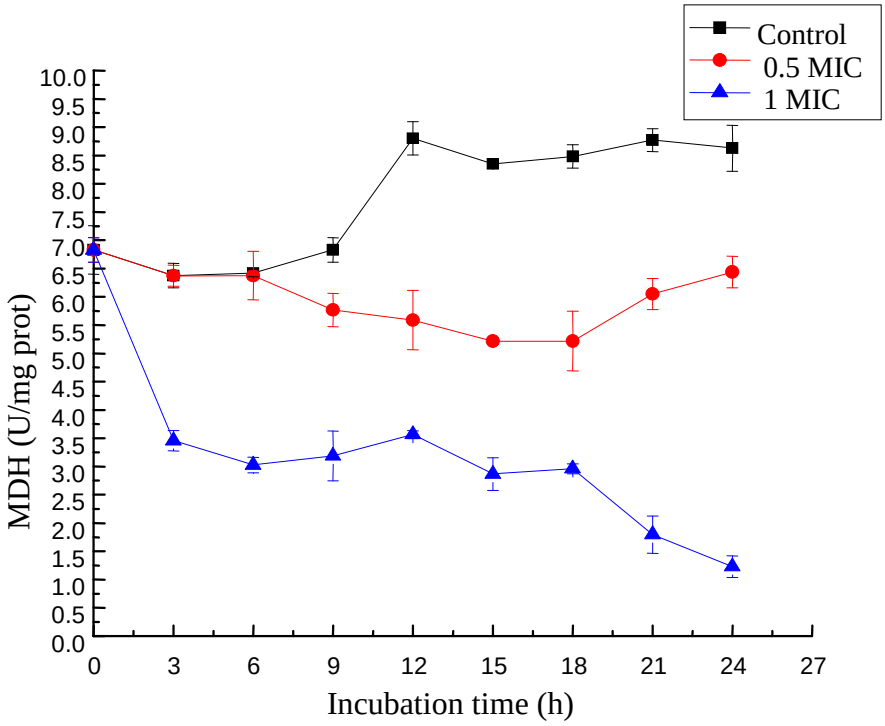

Fig.8: Effect of Cinnamon oil on MDH of *Rhizopus nigricans*

CONCLUSION

Cinnamon oil has antifungal activity and can change the mycelia morphology of *R. nigricans.* Further studies show that cinnamon oil can inhibit the ergosterol biosynthesis of the fungal membrane resulting in the damage of cell membrane, leading to cell content leak appearing as higher electric conductivity, $A_{260\ nm}$ and the amount of protein. And then the compounds of cinnamon oil can enter into the cells to decrease the activities of SDH and MDH of the tricarboxylic acid (TCA) cycle to affect the energy metabolism. The current study may provide some understandings of cinnamon oil as an antifungal agent.

Acknowledgements
This research was supported by Chinese Academy of Agricultural Sciences incremental projects and Science and Technology public service sectors of the Agriculture Ministry.

REFERENCES

[1] CA Nunes, *European Journal of Plant Pathology*, **2012**, 133(1), 181-196.
[2] KQ Liu; WH Yang; L Guo, *Journal of Zhongkai Agrotechnical College*, **2003**, 16(4), 1-6.
[3] BJ Wang; JF Liu; XH Fan; et al., *Food Machinery*, **2011**, 27(6), 52-54.
[4] S Burt, *International Journal of Food Microbiology*, **2004**, 94(3), 223-253.
[5] MM Tajkarimi; SA Ibrahim; DO Cliver, *Food Control*, **2010**, 21(9), 1199-1218.
[6] F Lu; Y Ding; X Ye; et al., *Agricultural Sciences in China*, **2011**, 10(9), 1482-1487.
[7] Y Xing; X Li; Q Xu; et al., *International journal of food science and technology*, **2010**, 45(9), 1837-1842.
[8] F Lv; H Liang; Q Yuan; et al., *Food Research International*, **2011**, 44(9), 3057-3064.
[9] J Tian; X Ban; H Zeng; et al., *Food Control*, **2011**, 22(12), 1992-1999.
[10] X Shao; S Cheng; H Wang; et al., *Journal of Applied Microbiology*, **2013**, 114(6), 1642-1649.
[11] S Paul; RC Dubey; DK Maheswari; et al., *Food Control*, **2011**, 22(5), 725-731.
[12] MM Bradford, Analytical Biochemistry, **1976**, 72(1), 248-254.
[13] J Tian; X Ban; H Zeng; et al., *PloS one*, **2012**, 7(1), e30147.
[14] RSN Brilhante; RAC de Lima; EP Caetano; et al, *Antimicrobial Agents and Chemotherapy*, **2013**, 57(5), 2167-2170.
[15] X Yao; X Zhu; S Pan; et al., *Food Chemistry*, **2012**, 132(4), 1883-1890.
[16] F Bakkali; S Averbeck; D Averbeck; et al., *Food and Chemical Toxicology*, **2008**, 46(2), 446-475.

[17] M Turgis; J Han; S Caillet; et al., *Food Control*, **2009**, 20(12), 1073-1079.
[18] MSA Khan; I Ahmad; SS Cameotra, *AMB Express*, **2013**, 3(1), 1-16.
[19] CF Carson; BJ Mee; TV Riley, *Antimicrobial Agents and Chemotherapy*, **2002**, 46(6), 1914-1920.
[20] X Sun; JC Wang; HT Li; et al., *Journal of Qingdao University (Natural Science Edition)*, **2005**,18(4), 41-45.
[21] J Tian; B Huang; X Luo; et al., *Food Chemistry*, **2012**, 130(3), 520-527.
[22] MS Xu; JP Chen; XC Shangguan, *Food Science*, **2005**, 26(4): 48-50.