

# Antifungal Activity of Osthole on *Microsporium canis* through Interfering with Biosynthesis of Fungal Cell Wall

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Liu *et al.*: Antifungal Effect of Osthole on *Microsporium canis*

Osthole extracted from *Cnidii Fructus*, a herb used in traditional Chinese medicine, was reported to possess multiple activities such as antibacterial, antiinflammatory, antitumor and insecticidal. However, its antifungal activity against *Microsporium canis* has not been investigated yet. The present study aimed to investigate the antifungal effect of osthole against *Microsporium canis*. The minimum inhibitory concentration and a growth inhibitory curve of osthole on *Microsporium canis* were according to the program of M38-A2 of Clinical and Laboratory Standards Institute. High-performance liquid chromatography analysis, aniline blue test and spectrophotometry were used to determine contents of ergosterol, 1,3- $\beta$ -D-glucan and chitin from *Microsporium canis*, respectively. The results indicated that minimum inhibitory concentration of osthole against *Microsporium canis* was 1.95  $\mu$ g/ml. High-performance liquid chromatography analysis demonstrated that there was no significant change in the content of ergosterol between the osthole and control group. Compared with the control group, the content of 1,3- $\beta$ -D-glucan and chitin in the cell wall of *Microsporium canis* was decreased. Taken together, this research showed that osthole could notably inhibit the growth of *Microsporium canis* and the antifungal activity was related to inhibiting the biosynthesis of 1,3- $\beta$ -D-glucan and chitin in the cell wall of *Microsporium canis*, making osthole a potential future antifungal agent.

**Key words:** Osthole, *Microsporium canis*, ergosterol, cell wall, 1,3- $\beta$ -D-glucan, chitin

*Microsporium canis* is one of the common and epidemic pathogenic fungi worldwide that can invade keratinized structures and infect hair, skin and nails of humans and animals. Furthermore, *M. canis* is zoonotic in nature and highly contagious to *Feline*, *Canis* and *Homo sapiens*<sup>[1,2]</sup>. The animals could be infected by *M. canis* during each season, notably, it has the highest incidence rate in the hot and damp seasons<sup>[3]</sup>. The pathogen causing 60 % of fungal skin diseases of *canis* and 98 % dermatophytes diseases of feline was found to be *M. canis*<sup>[4]</sup>. Once the animals infected by *M. canis*, the disease is hard to cure since it relapses easily and may generate cross-infection with other pathogens, which is characterized by increasing furfur, forming scab, exuding body fluid, pruritus and so on<sup>[5,6]</sup>.

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin (fig. 1), is a coumarin derivative extracted from *Cnidii Fructus*, a traditional Chinese medical herb<sup>[7]</sup>. Previous studies have showed that osthole could exert antibacterial, antiinflammatory, antitumor and insecticidal activities, relieve heart disorder, dilate blood

vessels and lower blood pressure<sup>[8-12]</sup>. A recent report showed that osthole could prevent the reproduction of *Alternaria* and *Cryptococcus neoformans*<sup>[13]</sup>. However, there is no investigation focusing on the antifungal activity and the possible targets of antifungal properties of osthole. Therefore, this preliminary assessment to investigate the inhibitory effect of osthole on *M. canis* was taken up.

## MATERIALS AND METHODS

Osthole (>98 % HPLC purity) was purchased from Nanning Innovative Pharmaceutical Technology Co., Ltd. (Guangxi, China). Caspofungin was obtained from New Pioneer Pharmaceutical Company (Shanghai, China). Terbinafine was purchased from Dalian Meilun

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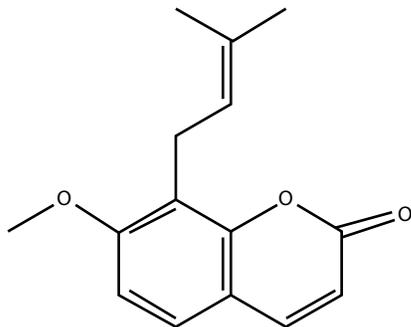


Fig. 1: Chemical structure of osthole

Bio-Technology Co., Ltd. (Dalian, China). Ergosterol, aniline blue and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Sabouraud dextrose agar (SDA) was purchased from Qingdao Hope BioTechnology (Shandong, China). RPMI-1640 liquid medium was purchased from Yuan Ye BioTechnology Co., Ltd. (Shanghai, China). Other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd., Shanghai Shi, China.

#### Fungi culture:

*Microsporum canis* (YUMCC Ym3099) was provided by the laboratory of fungi of Department of Clinical Veterinary Medicine at China Agricultural University in Beijing, China. *M. canis* was grown on SDA and incubated at 28° for 7 d, then picked single colony and cultured in Sabouraud dextrose broth. The concentration of *M. canis* was adjusted to 5×10<sup>5</sup> colony forming units (CFU)/ml using RPMI-1640 medium, and this suspension was used as inocula for the following inhibition assays.

#### Determination of minimum inhibitory concentration (MIC):

The MIC was determined with broth microdilution methods based on the reference document M38-A2 for yeast and filamentous fungi formulated by the Clinical and Laboratory Standard Institute (CLSI)<sup>[14]</sup>. Osthole, terbinafine and caspofungin were dissolved in DMSO and further diluted to final concentrations with RPMI-1640 medium. The final concentrations of osthole, terbinafine and caspofungin were 15.6, 0.8 and 0.16 µg/ml, respectively. The final DMSO concentration was ≤0.1 % (v/v). One hundred microliters of RPMI-1640 broth was added into each well of 96-well microtiter plates (Corning, NY, USA). Then either 100 µl osthole solution, terbinafine solution or caspofungin solution were added to the first well, and the multiple proportion dilution was conducted to obtain different

final drug concentrations. The above wells were supplemented with 100 µl spore suspension (5×10<sup>5</sup> CFU/ml) and for controls additional wells were only added to 200 µl RPMI-1640 medium, respectively. The plates were incubated for 7 d at 28° and performed in triplicate at each concentration. The absorbance at 620 nm was measured with a microplate reader (Bio-Rad, Foster, California, USA).

#### Inhibition curves of osthole, terbinafine and caspofungin on *M. canis*:

*M. canis* (5×10<sup>5</sup> CFU/ml) was treated with osthole, terbinafine and caspofungin at MIC concentrations during the logarithmic phase of its growth. After shaking for 0, 1, 2, 4, 8, 12, 24, 36, 48 and 72 h at 28°, the inhibitory effect of the compounds on *M. canis* was measured with the aid of a haemocytometer and growth inhibition curves were plotted.

#### High-performance liquid chromatography (HPLC) analysis:

*M. canis* was treated during logarithmic phase of its growth with osthole, terbinafine and caspofungin at MIC concentration for 24 h, respectively. After 7 d of incubation, *M. canis* hyphae was harvested by centrifugation at 1300 rpm for 15 min and washed twice with phosphate buffered saline at room temperature. The hyphae was collected, and resuspended in 15 ml saponifier and then incubated at 80° for 90 min. Then, 15 ml petroleum ether was added and incubated for 60 min. After being dried, the non-saponifiable extract was collected dissolved with methanol, and filtered with a Millipore filter of 0.22 µm. The aliquot was used for HPLC detection<sup>[15]</sup>.

The condition of chromatographic detection was 284 nm at 1 ml/min flow rate with 100 % methanol as mobile phase and column temperature at 35°<sup>[15]</sup>. Standard solution with different concentrations of ergosterol was prepared, and the sample was determined under the above chromatographic conditions. The standard curve of ergosterol was drawn by setting the concentration as abscissa and the peak area as the vertical. The resultant precision, stability and recovery of the HPLC methods were evaluated.

#### Measurement of 1,3-β-D-glucan:

The content of 1,3-β-D-glucan was determined using aniline blue assay<sup>[16]</sup>. Briefly, 50 µl suspensions of *M. canis* was added into 10 ml RPMI-1640 medium containing osthole or caspofungin at MIC

concentration, respectively. After incubation at 28° for 7 d, the *M. canis* was collected by centrifugation at 1300 rpm for 3 min, then washed twice with 0.1 M NaOH solution and freeze-dried for 16 h. A total of 5 mg *M. canis* was added into 250 µl 1 M NaOH solution. The mixture was blended for 30 s and incubated for 30 min at 52°. A volume of 50 µl the above mixture and 185 µl aniline blue (0.067 % aniline blue, 0.35 M HCl, 0.98 M glycine-NaOH, pH 9.5) were added into 96-well plates with triplicate and then incubated for 30 min at 52°. Absorbance was measured in a microplate reader (Bio-Rad, Foster, California, USA) at excitation/emission 410/458 nm.

#### Measurement of chitin:

The content of chitin was determined as previously described<sup>[14]</sup>. A volume of 50 µl suspensions of *M. canis* was added into 10 ml RPMI-1640 medium containing osthole or caspofungin at MIC concentration, respectively. After incubation at 28° for 7 d, the *M. canis* was collected by centrifugation at 1300 rpm for 3 min, then washed twice with 0.1 M NaOH solution and freeze-dried for 16 h. Briefly, 5 mg *M. canis* was resuspended in 3 ml KOH saturated solution, incubated for 1 h at 130°, added 8 ml 75 % ice-cold ethanol for 15 min in ice bath, and then mixed with a volume of 300 µl diatomite 545 suspension (13.3 %), and centrifuged at 2000 rpm for 5 min at 2°. The precipitate was washed once with 10 ml ice-cold ethanol (40 %), and then washed twice with 10 ml ice-cold water. The precipitate was resuspended in 500 µl sterile distilled water, 500 µl NaNO<sub>2</sub> (5 %) and 500 µl KHSO<sub>4</sub> (5 %), and then centrifuged at 2000 rpm for 2 min at 2°. After centrifugation, 150 µl supernatant was transferred to sterile tubes and then 450 µl sterile distilled water, 200 µl aqueous ammonia (12.5 %) and 200 µl 5 mg/ml 3-methylbenzothiazolinone-2-hydrazone were added and incubated for 3 min at 130°. The mixture was procedurally cooled down to room temperature, and 200 µl 0.83 % FeCl<sub>3</sub> was added, followed by incubation for 25 min at room temperature. Every sample was detected at 650 nm using spectrophotometer.

#### Statistical analysis:

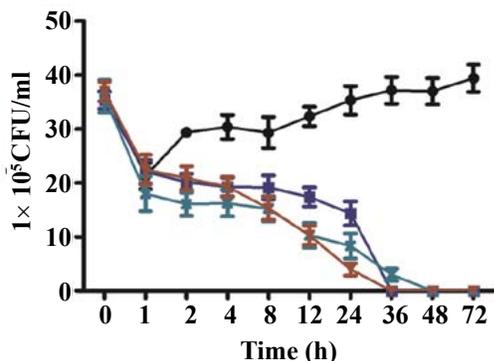
Results from a representative of three independent experiments were expressed as means±standard deviation (SD), and analysed using the one-way analysis of variance (ANOVA) followed by least significant difference test for multiple comparisons, and post-hoc test using SPSS 21.0 (SPSS, Inc., Chicago, IL, USA).

## RESULTS AND DISCUSSION

At present, synthetic drugs, including triazoles, polyenes and others, are being widely used for treating *M. canis* infection. With the wide application of these drugs as antifungal agents multidrug resistant fungi emerged on the clinical front<sup>[16]</sup>. Besides, the synthetic drugs also possessed certain deficiencies such as a poor curative effect on antifungal therapy<sup>[17]</sup>. Sensitivity to triazoles became reduced due to the deficiency of *Amb* binding sites, which could inhibit ergosterol biosynthesis<sup>[18]</sup>. Polyenes could inhibit the growth of fungi by interacting with ergosterol in cell membrane, but are ineffective on fungus without ergosterol in cell membrane<sup>[19]</sup>. Although the drugs designed for the cell wall of fungi such as caspofungin, micafungin and anidulafungin, have acquired corresponding progress, the drug species has been limited<sup>[17]</sup>. Therefore, in recent years natural product and active ingredient contained from those have attracted more and more attention, which could provide a new choice for the treatment of fungal diseases.

In this study, the results indicated that that MIC of osthole, terbinafine and caspofungin against *M. canis* were 1.95, 0.10 and 0.02 µg/ml, respectively. The number of viable fungi was not changed after treated with MIC of osthole, terbinafine and caspofungin for 1, 2, 4, 8, and 12 h. After being stimulated with osthole for 24 h, the number of viable fungi was significantly inhibited, and the effect of viable *M. canis* inhibition induced by osthole for 36 h was more serious. An obvious decrease in the number of viable fungi occurred after treatment with terbinafine and caspofungin for 24 h (fig. 2). The number of viable fungi significantly decreased with passage of time of exposure to osthole, terbinafine and caspofungin and declined to zero rapidly in the three groups, which suggested that osthole could absolutely inhibit the growth of *M. canis* within 36 h and exert the antifungal effect on *M. canis*. The results indicated that 1.95 µg/ml osthole and 0.02 µg/ml caspofungin could inhibit the growth of *M. canis* absolutely within 36 h, while the time of terbinafine inhibit the growth of *M. canis* absolutely was significantly greater than that of osthole. It was suggested that osthole has a good prospect in the treatment of infectious disease of *M. canis*.

Ergosterol, the main steroidal substance in the fungal cell membrane, can maintain integrity, viability and normal growth of fungi. In addition, ergosterol can



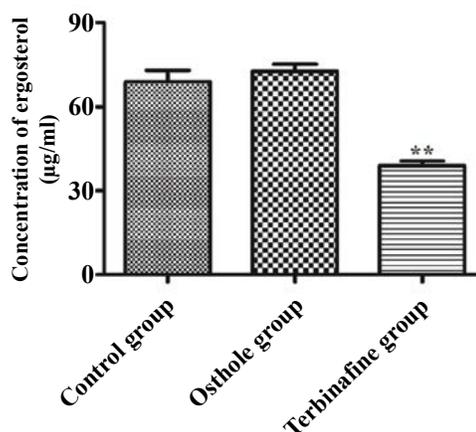
**Fig. 2: Inhibitory curves of osthole, terbinafine and caspofungin on *Microsporium canis***

The logarithmic phase of *M. canis* ( $5 \times 10^5$  CFU/ml) was exposed to MIC concentrations of osthole, terbinafine and caspofungin for 0, 1, 2, 4, 8, 12, 24, 36, 48 and 72 h, respectively. Data represent means  $\pm$  SD of 3 independent determinations and differences between mean values were assessed by one-way ANOVA. —●— Control group; —■— osthole group; —△— terbinafine group; —▽— caspofungin group

adjust cell membrane fluidity by combining with phospholipid<sup>[17,19]</sup>. The fungal cell might suffer fracture due to the lack of ergosterol<sup>[19]</sup>. Some agents exhibited antifungal activity by inhibiting biosynthesis of ergosterol or interacting with ergosterol, and destroying the cell membrane structure of fungi<sup>[17,20]</sup>. Terbinafine, a polyene antifungal drug, was set as a standard inhibitor of ergosterol synthesis. The content of ergosterol was detected by HPLC after the interaction of osthole with *M. canis*. The standard curve equation of ergosterol obtained was  $y = 1.7389x - 2.5539$  ( $r = 0.999$ ) under the liquid chromatograph condition. HPLC analysis results demonstrated that the content of ergosterol in osthole group and control group was significantly higher than that in terbinafine group, while no significant change was found in ergosterol content between osthole group and control group (figs. 3 and 4). The results demonstrated that osthole did not inhibit ergosterol biosynthesis in the cell membrane, which suggested that the antifungal activity of osthole could be related to the synthesis of cell wall.

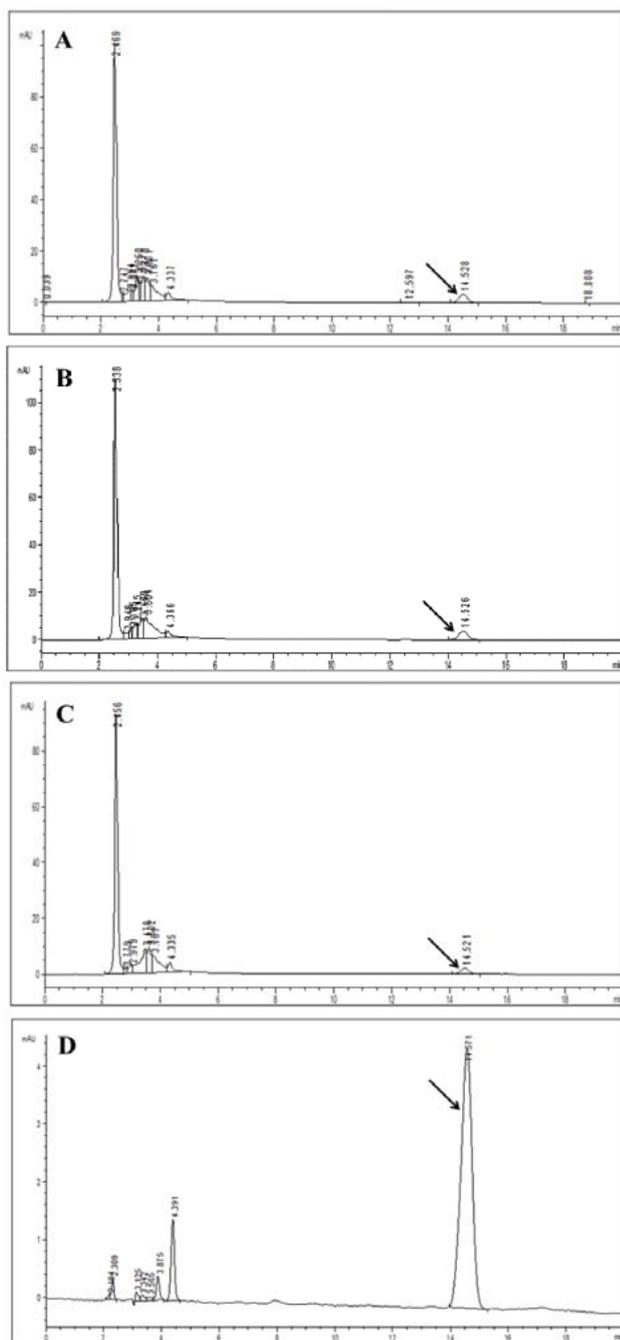
The fungi is endowed with a cell wall to maintain its cell shape and transmit the signals. The loss of cell wall structure or function can lead to the loss of cellular homeostasis, which ultimately leads to cell death. It was reported that the destruction of fungal cell wall might cause dissolution of fungal cells, which eventually cause fungal death<sup>[17,21]</sup>. Therefore, the fungal cell wall is the ideal targets of antifungal agents<sup>[22]</sup>. The main components of the fungal cell walls are 1,3- $\beta$ -D-glucan and chitin, which do not exist in mammalian cells. Some studies have shown that the transcription levels and activities of chitinase

and chitin synthase were significantly increased during the transformation of yeast to hyphal form of *Candida albicans*<sup>[23,24]</sup>. After cloning the chitinase gene and then performing site directed mutagenesis, the chitinase has obvious effect on both morphology and growth rate of mycelial and spore<sup>[25,26]</sup>. Moreover, chitinase plays an important physiological role in the building process of fungal cell wall, and its major function is to assist in synthesis of chitin synthase, and process and modify for synthesized chitin to achieve perfect space configuration of cell wall<sup>[27,28]</sup>. Thus, deficiency or insufficiency of chitinase will affect the normal growth of mycelium, and then influence the virulence of pathogenic fungi. 1,3- $\beta$ -D-glucan has effect on maintaining the normal shape of the fungal cell, when the fungal invade the deep part of body, and then it is processed by macrophage, the lots of 1,3- $\beta$ -D-glucan will be released; thus, 1,3- $\beta$ -D-glucan could be considered as a target molecule of fungal infection in clinical examination<sup>[14]</sup>. Besides, it is also reported that 1,3- $\beta$ -D-glucan, a fungal surface antigen, can cause a variety of immune responses in animal body<sup>[28,29]</sup>. In the past several decades, there appeared antifungal agent which can destroy cell walls by inhibiting the biosynthesis of chitin and 1,3- $\beta$ -D-glucan, such as echinocandins and caspofungins<sup>[28]</sup>. To investigate the effects of osthole on fungal cell wall synthesis, we carried out specific test to detect the content of fungal 1,3- $\beta$ -D glucan and chitin. The content of 1,3- $\beta$ -D-glucan in both osthole group and caspofungin group was significantly lower than that in control group,

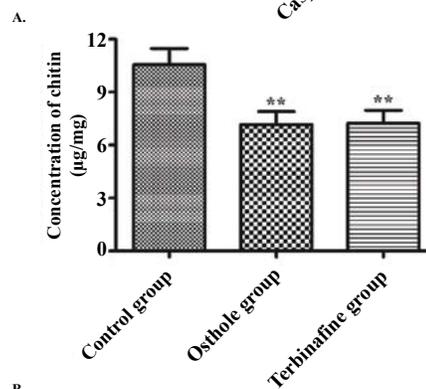
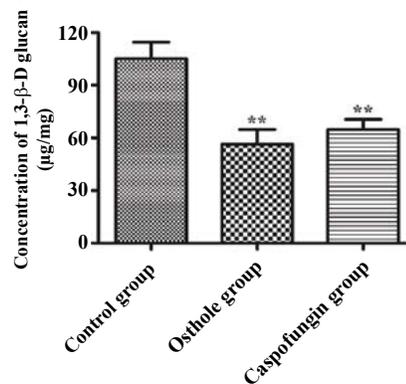


**Fig. 3: Effect of osthole on ergosterol in *Microsporium canis***  
Effect of osthole on ergosterol content of *M. canis* was measured using HPLC assay. The logarithmic phase of *M. canis* ( $5 \times 10^5$  CFU/ml) was treated with MIC concentration of osthole and terbinafine for 24 h, respectively. Data represent means  $\pm$  SD of three separate experiments and differences between mean values were assessed by one-way ANOVA. \*\* $P < 0.01$  indicates the significant difference compared with control group

while no significant change was found in the 1,3- $\beta$ -D-glucan content between osthole group and caspofungin group (fig. 5A). The data demonstrated that the content of chitin in both osthole group and caspofungin group was significantly decreased compared with the control group, while there was no significant difference in the content of chitin between osthole group and caspofungin group (fig. 5B). The results of this study indicated that the osthole has apparent inhibitory effect



**Fig. 4:** HPLC chromatogram of ergosterol in *Microsporium canis*  
**A:** Control group; **B:** osthole group; **C:** terbinafine group; **D:** ergosterol standard material.



**Fig. 5:** Effect of osthole on 1, 3- $\beta$ -D-glucan (A) and chitin (B) in *Microsporium canis*

Data represent means $\pm$ SD of three separate experiments and differences between mean values were assessed by one-way ANOVA. \*\*P<0.01 indicates the significant difference compared with control group

both on chitin and 1,3- $\beta$ -D-glucan biosynthesis. Thus, osthole has stronger inhibition on inherent components of the *M. canis* cell wall.

In summary, our investigation demonstrated that osthole exerted an antifungal effect on *M. canis* by decreasing the content of 1,3- $\beta$ -D glucan and chitin, thereby interfering with the biosynthesis of fungal cell wall, and these results make a strong case that osthole could be a potential antifungal in place of the existing drugs.

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**Conflict of interest:**

The author declares that there is no conflict of interest regarding the publication of this paper.

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