Effects of Two Curcuminoids on *Candida albicans*

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Abstract: **Objective** To investigate and compare the action of curcuminoids on the causal pathogens of *Candida albicans* growth. **Methods** The effects of curcumin (CUR) and demethoxycurcumin (DMC) on *C. albicans* growth were first investigated and compared by microcalorimetry coupled with multiple analytical methods. The quantitative thermo-kinetic parameters obtained from these curves were analyzed to show difference of the actions. **Results** By analyzing the main parameters screened from principal component analysis together with 50% inhibiting concentration values, it was demonstrated that both CUR and DMC showed good antifungal activities and CUR was stronger. It was further concluded from structure-activity relationship that the existence of methoxy group might enhance lipophilicity of the mother nucleus, which made it easier for the molecular to enter into the cell membrane of fungi to inhibit its growth. **Conclusion** This study provides a new method for screening new antifungal agents with high efficacy and low toxicity. Meanwhile, it contributes to the application of curcuminoids as food additive, colorant, and drug. Microcalorimetry is real-time, online, and dynamic, and it could be used to characterize the subtle difference among the effects of synthetic and natural products on the vital process of fungi.

Key words: antifungal agents; *Candida albicans*; comparative study; curcuminoids; microcalorimetry
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Introduction

In recent years, there has been an increasing threat to life caused by fungous infection, and fungi have emerged as the fourth most-common pathogens isolated in nosocomial bloodstream infections, nearly 40% of which prove fatal (Morrell, Fraser, and Kollef, 2005; Eckmanns, Ruden, and Gastmeier, 2006). Candidiasis is known as the most common invasive fungal infection in critically ill non-neutropenic patients (Eggimann, Garbino, and Pittet, 2003). Among the various species, *Candida albicans* (Robin) Berkh. is the most causative agent associated with serious fungal infection, accounting for more than 90% of cases (Douglas, 2003). *C. albicans* could cause galactic damage to people's health because of its disoperation to skin, mucosa, and internal organs (Zhao *et al*, 2010; Kong *et al*, 2011). But the management of candidiasis faces a number of problems including limited number of effective antifungal agents, toxicity of the available antifungal agents, resistance of candidiasis to commonly used antifungal agents, relapse of candidiasis infections, and the high cost of antifungal drugs (Mustafa *et al*, 1999; Klepser, 2001; Khan, Chandy, and Metwali, 2003; Runyoro *et al*, 2006). Therefore, it is necessary to screen for new antifungal agents with high efficacy and low toxicity.

Curcuminoids are isolated from many species of plants, which are widely favored by virtue of their special taste (Eigner and Scholz, 1999). Meanwhile, curcuminoids have been used for a long time as food

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additives and colorant in India, China, and Southeast Asia by means of its brilliant yellow color, purely natural, and pollution-free quality. Nowadays, curcuminoids are widely concerned in pharmaceutical science, and a series of drugs have been developed possessing a broad spectrum of biological actions e.g. antibacterial (Mahady *et al*, 2002; Han and Yang, 2005), antifungal (Banerjee and Nigam, 1978), anti-oxidative (Kunchandy and Rao, 1990; Cohly *et al*, 1998; Di *et al*, 2010), and anticarcinogenic activities (Aggarwal, Kumar, and Bharti, 2003; Chattopadhyay *et al*, 2004). More importantly, it has been proved that curcuminoids are well tolerated at a very high dose without any toxic effects (Chainani-Wu, 2003; Anand *et al*, 2007; Goe, Kunnumakkara, and Aggarwal, 2008).

Despite the widely concerned antifungal effects of curcuminoids, little attention has been paid to their structure-activity relationship (Zhang *et al*, 2008) or mechanisms (Salunke *et al*, 2004). In this study, we chose curcumin (CUR) and demethoxycurcumin (DMC), two main constituents in curcuminoids (Ruby *et al*, 1995), to investigate their effects on *C. albicans* growth. The results will not only give us the information about the effects of CUR and DMC, but also provide evidence for the study on structure-activity relationship. However, this work will be hard to process unless an appropriate method is applied, providing as much information as possible.

Traditional methods, such as disc-diffusion (Huang *et al*, 2009), broth macrodilution (Hazra *et al*, 2004; Chen *et al*, 2006), agar dilution (Mohamed *et al*, 2005), and cup-plate methods (Zhou, Pan, and Li, 2009), are all invasive so that they require sampling of the microbiological sample at specific time points and bacterial resistance, even a high likelihood of cross-resistance arises soon after the antibacterial drugs are widely used in the community (Coates *et al*, 2002). For this reason, it is of great significance to find a more precise and suitable approach. Microcalorimetry, a non-destructive technique, has long been used to study antimicrobial activities of drugs and other materials (Phipps and Mackin, 2000; Wadsö, 2002; Wang *et al*, 2010). It permits the online test of bioactivity screening and could offer a lot of important information about the process of microbial cell growth, which could not be obtained by other techniques.

Therefore, in this study, microcalorimetric technique was applied to investigating the effects of CUR and DMC on *C. albicans* growth. By analyzing the heat-flow power (HFP)-time curves of *C. albicans* growth in the presence of CUR and DMC and processing the quantitative thermo-kinetic parameters obtained from the growth curves with multiple analytical methods, the antifungal effects of CUR and DMC on *C. albicans* growth were characterized and compared. Furthermore, the possible structure-activity relationship of curcuminoids was discussed. This study provided a simple, fast, and sensitive method for the investigation of antifungal activity of curcuminoids and other materials. The results of this study would be valuable for the application of curcuminoids as a healthy food additive, colorant, and drug in our daily lives.

Materials and methods

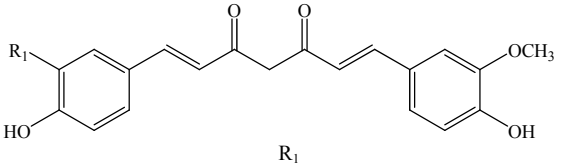
Fungal strains and culture conditions

Strain *C. albicans* (CCTCC AB64550) was provided by China Center for Type Culture Collection, Wuhan University (China). The broth culture medium (pH 7.0－7.2, 1000 mL) contained peptone (10 g), beef extract (6 g), and NaCl (5 g). The volume of the container was 100 mL, and the volume of the culture medium was 25 mL. The culture medium was sterilized in high pressure steam at 121 ℃ for 30 min. *C. albicans* was inoculated in conical flask with 25 mL broth culture medium by order, and then incubated in the shaker for 8 h at 37 ℃. The rotation speed of incubator shaker was 120 r/min. The conical flask was enveloped with a cotton plug, so that there was enough oxygen.

C. albicans was grown in the Luria-Bertani (LB) culture medium (pH 7.0－7.2, 1000 mL) prepared from peptone (10 g), yeast extract (5 g), and NaCl (5 g). The medium was sterilized by autoclaving at 121 ℃ for 30 min, and stored in a refrigerator at 4 ℃.

Chemicals

CUR and DMC were purchased from Beijing Yasser Co., Ltd. (China). The purity of them was determined to be over 95% by UPLC analysis. And their structures were given in Fig. 1. Dimethyl sulfoxide (DMSO) was chosen as a solvent for preparing the original solution of the two compounds. All the other chemicals were of analytical grade.



	R_1
CUR	OCH_3
DMC	H

Fig. 1 Chemical structures of CUR and DMC

Microcalorimetric studies

The experiments were performed at 37 ℃ using TAM III isothermal microcalorimeter (Thermometric AB, Sweden) with ampoule method. *C. albicans* was inoculated in 100 mL LB medium, initially with the density of 1×10^6 colony forming units (CFU)/mL. *C. albicans* suspension (2 mL) was added into each sterilized 4 mL glass ampoule. CUR and DMC were diluted in order in 2 mL DMSO, then the solution at different concentration was introduced into this suspension. Eventually, each ampoule containing different concentration of CUR, DMC, and *C. albicans* suspension was sealed up and put into the equilibrium position of the calorimeter block. After about 15 min (the ampoules reached equilibrium in the air), the ampoules were lowered into the measuring position of the calorimeter block. After another 45 min (the temperature of the ampoules reached 37 ℃), the HFP-time curves were recorded until the recorder returned to the baseline. All data were continuously collected using the dedicated software package (PicoLog TC-80, TA Corporation, USA).

Similarity analysis (SA)

The SA for HPLC fingerprints of traditional Chinese medicine from different sources was carried out (Chen *et al*, 2008). The thermogenic curves of *C. albicans* growth affected by different concentration of CUR and DMC were investigated by their similarities, so as to intuitively and quickly find the influence of the compounds on the fungal growth. In this study, the correlation coefficients of similarity among the thermogenic curves of *C. albicans* growth with and without CUR and DMC were calculated using the cosine method.

Results

Choosing the appropriate solvent concentration

In this study, CUR and DMC were firstly dissolved in DMSO, and then diluted with the LB culture. Different DMSO concentration in the solvent was investigated, i.e., 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%. When the concentration of DMSO exceeded 0.3%, DMSO could be well distributed in the final solution. Meanwhile, we investigated the effect of DMSO at different concentration on *C. albicans* growth to eliminate the influence of the solvent. The results showed that with the increase of the DMSO concentration, especially above 0.3%, from the HFP-time curves, all the peak height and the appearance time of second peak declined gradually. While the concentration was less than 0.3%, the influence could be neglected. By duplicated experiments and comparison between CUR and DMC, the DMSO concentration was defined within 0.3% during the experiments.

HFP-time curves of *C. albicans* growth

The growth thermogenic curves of *C. albicans* at 37 ℃ in the absence of any substance were shown in Fig. 2. The HFP-time curves show the total metabolism profile of *C. albicans*, and each could be divided into two stages (stages 1 and 2) and the following five phases, i.e., lag phase (A—B), the first exponential growth phase (B—C), transition phase (C—D), the second exponential growth phase (D—E), and decline phase (E—F).

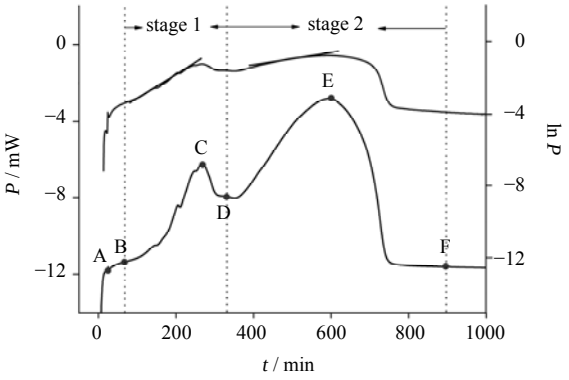


Fig. 2 HFP-time curves of *C. albicans* without any substance

Similarly, the HFP-time curves of *C. albicans* growth in the presence of different concentration of CUR and DMC were recorded and the corresponding curves were shown in Fig. 3. The concentration of CUR or DMC of a—f in this profile was increasing by orders, and the concrete concentration for all the experiments was shown in Table 1. We could easily conclude from Fig. 3 that the shapes of the HFP-time curves changed

regularly along with the increase of the concentration of CUR or DMC. As for Fig. 3A, small changes of the curves shapes could be delineated when added into low concentration of CUR, while visible changes could be observed with high concentration of CUR.

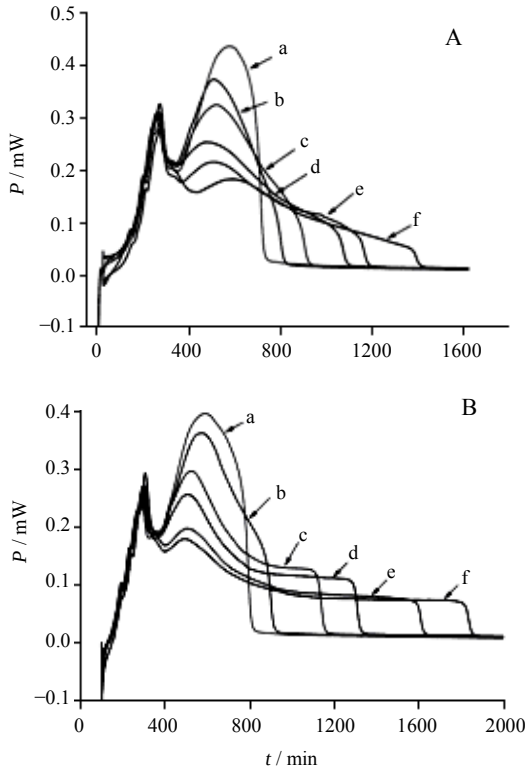


Fig. 3 HFP-time curves of *C. albicans* growth at 37 ℃ in presence of different concentration of CUR (A) and DMC (B)

Quantitative thermo-kinetic parameters for *C. albicans* growth

The HFP-time curve of *C. albicans* growth could be delineated with the following equation (Xie *et al*, 1988): $P_t = P_0 \exp(kt)$ or $\ln P_t = \ln P_0 + kt$, where P_0 and P_t represented the HFP at time 0 and t (min), respectively. Using this equation, the growth rate constants (k_1 and k_2) of the first and second exponential phases for *C. albicans* growth at 37 ℃ in the absence of any substance were calculated by analyzing the data of the first and second highest peaks. In order to test the reliability of the microcalorimetry, we repeated the experiments for eight times for the bacteria in the absence of any substance and obtained good reproducibility. Afterwards, the quantitative thermo-kinetic parameters, such as the HFP of the first and the second highest peaks (P_1 and P_2), the appearance time of the first and second highest peaks (t_1 and t_2), the heat output in stages 1 and 2 ($Q_{sta,1}$ and $Q_{sta,2}$), and the total heat output (Q_t), were obtained from the HFP-time curves of *C. albicans* growth affected by different concentration of CUR and DMC (Table 1).

SA

The similarities among the HFP-time curves of *C. albicans* growth with and without different concentration of CUR or DMC were calculated on the correlative coefficient of original data in Table 1 with cosine method using software of Microsoft Excel 2003.

Table 1 Quantitative thermo-kinetic parameters for *C. albicans* growth at 37 ℃ affected by CUR and DMC

Groups	C / (μg·mL^{-1})	k_1 / min^{-1}	R^a	t_1 / min	P_1 / mW	k_2 / min^{-1}	R^a	t_2 / min	P_2 / mW	$Q_{sta,1}$ / J	$Q_{sta,2}$ / J	Q_t / J
control	0	0.014 01	0.9995	268.4	0.2897	0.004 71	0.9966	595.3	0.4586	2.21	9.02	11.23
CUR	20	0.013 94	0.9986	270.8	0.2879	0.004 69	0.9956	580.0	0.4353	2.28	8.90	11.18
	60	0.013 89	0.9938	256.4	0.3068	0.004 01	0.9983	508.6	0.3724	2.18	8.79	10.97
	70	0.013 85	0.9922	262.3	0.3062	0.003 75	0.9940	521.6	0.3235	2.35	8.78	11.13
	100	0.013 97	0.9991	267.7	0.3098	0.002 82	0.9930	473.7	0.2725	2.29	8.89	11.18
	120	0.013 88	0.9979	275.8	0.2792	0.002 43	0.9980	510.9	0.2198	2.29	8.87	11.16
	200	0.013 99	0.9919	274.1	0.3255	0.001 18	0.9959	596.9	0.1419	2.30	8.92	11.22
DMC	40	0.014 07	0.9974	267.2	0.2838	0.004 32	0.9981	597.1	0.4169	2.20	9.01	11.21
	60	0.014 24	0.9997	268.2	0.2896	0.004 14	0.9962	592.8	0.4037	2.22	8.92	11.14
	80	0.014 15	0.9998	271.4	0.2933	0.003 57	0.9956	593.8	0.3577	2.26	8.91	11.17
	100	0.013 98	0.9983	272.7	0.2909	0.003 44	0.9938	599.5	0.3473	2.29	8.85	11.14
	300	0.014 41	0.9994	266.0	0.3045	0.002 01	0.9963	592.3	0.2384	2.28	8.95	11.23
	400	0.014 50	0.9988	272.3	0.2932	0.001 60	0.9939	595.6	0.2103	2.26	8.96	11.22

The thermogenic curves which showed the growth of *C. albicans* in the absence of any substance were regarded as the reference, and the thermogenic curves in the presence of different concentration of CUR and DMC were compared accordingly with them. The corresponding data set of similarity is shown in Fig. 4.

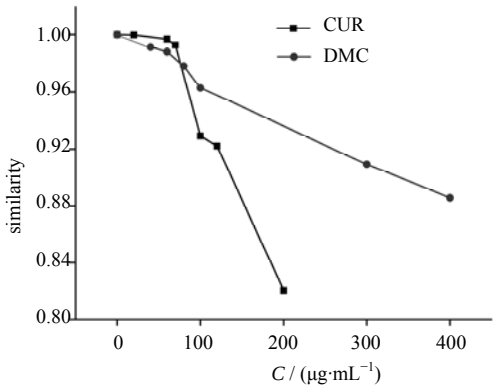


Fig. 4 Delineation of similarities of thermogenic curves

It could be illustrated from Fig. 4 that CUR and DMC of different concentration had varied effects on *C. albicans* growth. The decrease of the correlation coefficient also illustrated that the antifungal effects were enhanced with increasing the concentration of CUR and DMC, and we could roughly conclude from Fig. 4 that the antifungal activity of CUR on *C. albicans* was stronger than that of DMC. However, by analyzing the multivariate variables in Table 1, we might notice that the nine parameters have different change trends (increasing and decreasing) with the increase of concentration of CUR and DMC, making it difficult to accurately compare the antifungal effects of CUR and DMC. So, it was necessary to extract the main parameter(s) that played the most important role in evaluating the antifungal effect.

Evaluation of the antifungal activity

Returning to the two main parameters k_2 and P_2 in Table 1, no significant or major differences were observed between them. For this reason, the box plot in Fig. 5 of k_2 and P_2 for CUR and DMC provided some help. This plot markedly showed the distribution of the k_2 and P_2 data for CUR and DMC. It could be perspicuously seen that the minimum and maximum values, the median, 25% and 75% quartile values of k_2 and P_2 for DMC were larger than those of CUR, indicating that the antifungal effect of CUR was stronger than that of DMC.

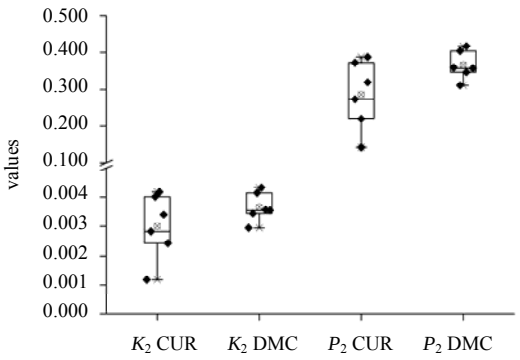


Fig. 5 Box plot of k_2 and P_2 for CUR and DMC

This plot was made using software of Origin 8.0, demonstrating the minimum and maximum values, the median, 25% and 75% quartile values and the range

The inhibitory ratios (I) of CUR and DMC on *C. albicans* were obtained from k_2, which could describe the change tendency of the antifungal efficacy among different concentration of CUR and DMC. Finally, the 50% inhibitory concentration (IC_{50}) of CUR and DMC was calculated and valued as 134.2 and 274.5 μg/mL, respectively. Good linear correlation between k_2, P_2, I, and the concentration was obtained (Fig. 6).

Discussion

The results of this study were consistent with the research on the anti-oxidative effect of curcuminoids, sharing the same active group (Unnikrishnan and Rao, 1995; Song *et al*, 2001). Although the types of curcuminoids concerned in this study were limited or complete information on structure-activity relationship could not be obtained, this study provided a brand new method for evaluating the antifungal effect of drugs. In addition, this method was online and accurate, which could characterize minor variations of the antifungal activity among different drugs. Furthermore, it could profit the development of antifungal agents which are more effective and safer by means of structural modification.

The shapes, chemical components, and physiological nature of different individuals of bacteria in exponential phases were all coincident. In exponential phases, the metabolism of bacteria is productive, the growth of bacteria is fast, and the reproductive cycle is stationary, which makes the bacteria good materials in these phases for the investigation of analytic metabolism of microbes.

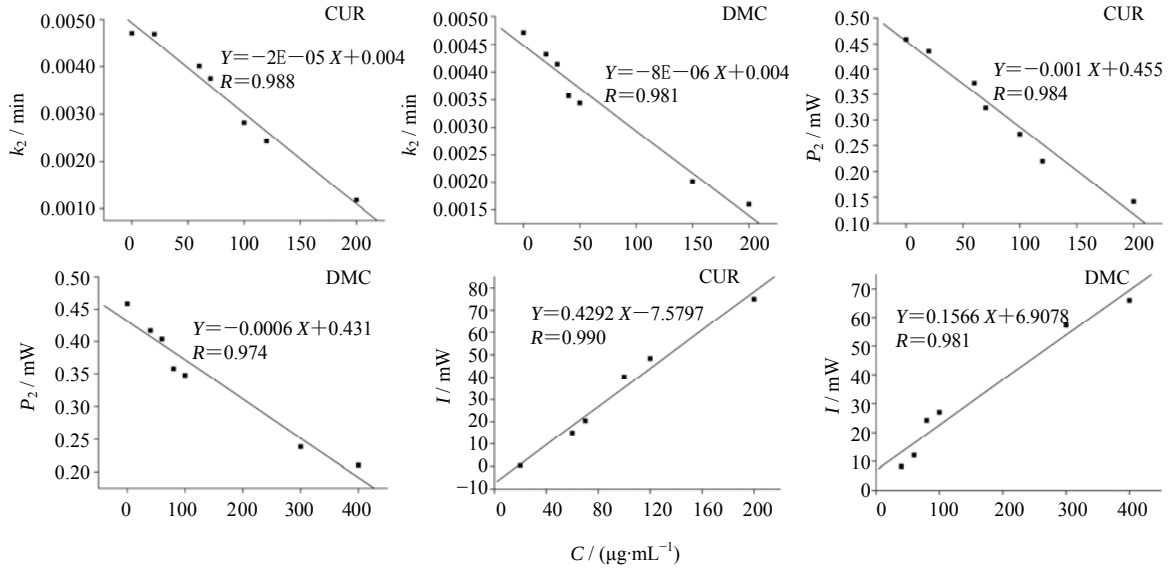


Fig. 6 Relationship between k_2, P_2, I_2, and the concentration of CUR and DMC

Moreover, the shape, color, and bioactivity of bacteria are typical and sensitive to the effect of environmental factor, so that these stages of bacteria are the best object for the research of bacterial characters. Therefore, in this study, the atlas and data within the exponential stages were mainly analyzed (Brunner *et al*, 2008).

The growth and metabolism of fungi were observed by microcalorimetry, which was real-time, online, and dynamic. This method could be used to characterize the heat production during the vital process of fungi, including stagnation, exponential growth, and death (all the nutrient substances were consumed) (Huang *et al*, 2009; Kong *et al*, 2009; Zhou, Pan, and Li, 2009). By analyzing different growth stages of the HFP-time curves and extracting and comparing the characteristic parameters, the condition of the fungi could be obtained (Bansal, Singh, and Garg, 2009). When fungi were exposed to drugs, their HFP-time curves would change, which could reflect the influence of drugs on the growth of fungi.

Not only could HFP-time curves offer the information of anti-bacterial/fungal activity, but also provide the toxicity and safety information of drugs (Kong *et al*, 2010). For instance, the category and amount of microbes throughout the manufacture and utilization of food and injection were rigorously handled. Microcalorimetry, with the specificity of fingerprint, was able to provide a specific HFP-time curve for each category of microbes. In addition, bio-thermal has a high sensitivity (the accuracy of the temperature of TAMIII could reach 0.0001 ℃, and the accuracy of the heat power could reach 10^{-9} W) (Yao *et al*, 2007), which is suitable to be applied in the control of the microbes in food and drug. It was further estimated that this method was superior in the sterility test of injections.

Curcuminoids, as food additives, industrial colorant, and drug, will win approval if tested to be with broad-spectrum antifungal effects. For example, food additives that comprise curcuminoids could be stored longer; Cloths that dyed with curcuminoids will defend the infection caused by fungal; Patients who have curcuminoids food during the curing process will reduce the danger of fungous infection. Curcuminoids have a wide safety margin, while no toxicity or adverse reaction has ever been reported even when giving a large dose of curcuminoids in the clinic (Anand *et al*, 2007; Goe, Kunnumakkara, and Aggarwal, 2008). Given the broad-spectrum antifungal activity, we reasoned that curcuminoids could be a great ingredient for the development of new antifungal drugs.

Conclusion

The antifungal effects of curcuminoids were first investigated in this study, and the antifungal effect of CUR was stronger than that of DMC. We have inferred from the structure-activity relationship that 3-methoxy group, which enhanced the antifungal effect of curcuminoids, might increase the lipophilicity of

curcuminoids and make it easier for drugs to enter into cells of fungi. This study could provide an online and sensitive method for screening new antifungal agents with high efficacy and low toxicity. Meanwhile, this work contribute to the application of curcuminoids as food additive, colorant, and drug.

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