

# Antidermatophytic and Protease-inhibiting Activities of Zerumbone: A Natural Sesquiterpene from the Rhizome of *Zingiber zerumbet* (L.) Roscoe ex J.E; Smith

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## ABSTRACT

**Context:** Due to increase in the number of patients with impaired immunity, incidence of dermatophytoses has increased considerably. Antidermatophytic agents with anti-inflammatory and protease-inhibiting activities will help in restricting inflammatory response associated with dermatophytoses. **Aims:** The present study aims to evaluate antidermatophytic and protease-inhibiting activities of zerumbone. Cytotoxicity was tested using Chang liver cell line as a preliminary step in toxicity study. **Methods and Materials:** Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of zerumbone purified from the rhizome of *Zingiber zerumbet* were determined against *Epidermophyton floccosum* var. *nigricans*, *Microsporum canis*, *Microsporum gypseum*, and *Trichophyton rubrum*. MIC was determined according to Clinical and Laboratory Standards Institute (CLSI) method M38-A2. Protease-inhibiting property was tested using trypsin as the enzyme. *In vitro* cytotoxic effect was studied using the MTT assay. **Results:** MIC of zerumbone was 8 mg/L against *E. floccosum* and *M. canis* and 16 mg/L for *M. gypseum* and *T. rubrum*. MFC of zerumbone was 64 mg/L against *E. floccosum* and *M. canis* and 128 mg/L for *M. gypseum* and *T. rubrum*. Zerumbone exhibited remarkable protease-inhibiting activity. In the MTT assay,  $IC_{50}$  values were 150 and 0.31  $\mu$ g, respectively, for zerumbone and reference drug. **Statistical Analysis Used:** For protease inhibition, assay and cytotoxicity assay control and tests were done in triplicate and the results are expressed as mean  $\pm$  SD, where  $n = 3$ . **Conclusions:** Zerumbone is a novel candidate for use in dermatophytoses therapy because of the combined antifungal, anti-inflammatory (unpublished results), and protease-inhibiting properties. Cytotoxicity of zerumbone was found to be very low compared with the reference drug.

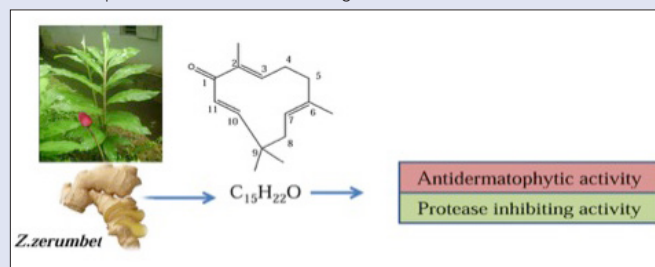
**Key words:** Epidermophyton, microsporum, protease, trichophyton, trypsin

## KEY MESSAGES

- Zerumbone possesses antidermatophytic, anti-inflammatory, and protease-inhibiting activities. Hence, it is a novel candidate for the development of new antidermatophytic drug.
- Cytotoxicity of zerumbone against Chang liver cell line was found to be very low compared with the reference drug doxorubicin.

## SUMMARY

- Zerumbone isolated from the rhizome of *Zingiber zerumbet* exhibited antidermatophytic activity against *E. floccosum* and *M. canis* (MIC 8 mg/L) and *M. gypseum* and *T. rubrum* (MIC 16 mg/L).
- Zerumbone exhibited remarkable protease-inhibiting activity.
- Zerumbone is a novel candidate for the development of new antidermatophytic drug.
- Cytotoxicity of zerumbone against Chang liver cell line was found to be very low compared with the reference drug doxorubicin.



**Abbreviations used:** CFU: colony forming unit, CLSI: Clinical and Laboratory Standards Institute, COX: cyclooxygenase, DMSO: dimethyl sulphoxide, EDTA: ethylene diamine tetra acetic acid, FTIR: Fourier transform-infrared spectroscopy, HPLC: high-performance liquid chromatography, LOX: lipoxygenase, IMTECH: Institute of Microbial Technology, LCMS: liquid chromatography mass spectrometry, MTT: 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTCC: microbial type culture collection, MFC: minimum fungicidal concentration, MIC: minimum inhibitory concentration, MPO: myeloperoxidase, NMR: nuclear magnetic resonance spectroscopy, PAR: proteinase-activated receptor, PBS: phosphate-buffered saline, TCA: trichloro acetic acid

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## INTRODUCTION

Dermatophytes are a group of closely related pathogenic fungi that infect humans and other animals. They invade keratinized structures like skin, hair, and nails and the infections produced are known as dermatophytoses.<sup>[1]</sup> Dermatophytic infections are usually cutaneous and noninvasive. But different types of allergic and inflammatory reactions are induced in the host by the presence of these fungi and their metabolic products resulting in unsightly, itchy, and treatment-resistant lesions. Hence, use of agents with antifungal as well as anti-inflammatory activity

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is the emerging trend in the treatment of dermatophytoses especially for topical applications. It has been reported that serine protease secreted by dermatophytes causes itching.<sup>[2]</sup> Therefore, it is anticipated that antifungal agents with protease-inhibiting property can provide rapid symptomatic relief in dermatophytoses.

In this study, we have evaluated the antidermatophytic and protease-inhibiting activities of zerumbone isolated from the rhizome of *Zingiber zerumbet*. *In vitro* cytotoxic effect of the compound was also studied using the MTT assay using Chang liver cell line. *Zingiber zerumbet* is a medicinal plant belonging to the family Zingiberaceae. Its rhizome is used as food-flavouring agent and appetizer in various cuisines, while the rhizome extract is used in traditional medicine for various ailments. The present study is based on the observation of the antidermatophytic and protease-inhibiting activities of the rhizome of *Z. zerumbet*.<sup>[3]</sup>

Zerumbone is a monocyclic sesquiterpene found abundantly in the rhizome of *Z. zerumbet*. Zerumbone has been reported to possess anticancer,<sup>[4,5]</sup> anti-inflammatory,<sup>[4,6]</sup> immunomodulatory,<sup>[7]</sup> antinociceptive,<sup>[8]</sup> antimicrobial activity against plant pathogens,<sup>[9]</sup> and antioxidant<sup>[4]</sup> activities. This study is the first report on the antidermatophytic and protease-inhibiting activities of zerumbone.

## MATERIALS AND METHODS

### Microorganisms and media

The following dermatophytes were procured from MTCC, Institute of Microbial Technology (IMTECH), Chandigarh.

- *Epidermophyton floccosum* var. *nigricans* (MTCC 613)
- *Microsporum canis* (MTCC 2820)
- *Microsporum gypseum* (MTCC 2819)
- *Trichophyton rubrum* (MTCC 296)

Cultures of *M. gypseum* and *M. canis* were maintained in Sabouraud dextrose agar and *E. floccosum* and *T. rubrum* in Emmons modification of Sabouraud dextrose agar (HiMedia). The purity of standard cultures was tested by examining the morphology of macroconidia and shape and disposition of microconidia by lactophenol cotton blue staining and by macroscopic appearance and pigmentation of the colony on Sabouraud dextrose agar.<sup>[10]</sup>

### Isolation, purification, and characterization of zerumbone

Isolation and purification of zerumbone was done according to the method of Abdul *et al.*, with slight modification.<sup>[5]</sup> Petroleum ether fraction of the methanolic extract of the dried and powdered rhizome of *Z. zerumbet* was concentrated and subjected to column chromatography using silica gel (60-120 mesh). Zerumbone was eluted using the mobile phase hexane and ethyl acetate in the ratio 8.5:1.5 and was further purified by repeated recrystallization from petroleum ether.

Purity of the isolated crystals was checked by high-performance liquid chromatography (HPLC) and then the crystals were subjected to Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and liquid chromatography-mass spectrometry (LC-MS) analyses for characterization.

HPLC analysis was performed using Shimadzu Prominence LC 20AP system equipped with pump LC 20AP, UV-VIS detector SPD 20A/20AV and LC solution software. Column used was Enable RP C18 G250 (250 mm × 4.6 mm × 5 μm, 120 Å). Seventy percent acetonitrile was used as the mobile phase at the flow rate of 1 mL/min. FT-IR spectrum of the compound was recorded in Attenuated Total Reflection (ATR-FTIR) mode (FTIR Perkin-Elmer Spectrum 400). <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of the compound were recorded using CDCl<sub>3</sub> as the solvent

system (Bruker AV 400 MHz NMR spectrometer). LC-MS analysis was done using Acquity UPLC BEH C18 column (LC-MS/MS system (Walters) fitted with Acquity model UPLC and Xevo G2 QTOF Mass spectrometer). Binary gradient of acetonitrile and water containing 0.1% formic acid—5:95 (0-5 min), 95:5 (5-6.5 min), and 5:95 (6.5-10 min)—was used as the mobile phase at the flow rate of 0.25 mL/min. MS/MS analysis was performed using Quantitative Time of Flight (Q TOF) analyser. The spectrum was taken in positive ionization mode.

### Antidermatophytic activity test: determination of minimum inhibitory concentration and minimum fungicidal concentration

The minimum inhibitory concentration (MIC) of zerumbone was determined according to CLSI method (Clinical and Laboratory Standards Institute) M38-A2, with slight modification.<sup>[11]</sup> Sabouraud dextrose broth (HiMedia) was the medium used instead of RPMI 1640. Zerumbone was dissolved in dimethyl sulphoxide (DMSO) and dilutions were prepared in sterile broth so that, after subsequent addition of inoculum, the final drug dilutions were 0.5-256 mg/L (doubling dilutions). Griseofulvin (Sigma) was used as the reference drug.

Conidia were harvested from 21 days old (45 days for *E. floccosum*) culture on Sabouraud dextrose agar slants and counted in a haemocytometer. Concentration of conidia was then adjusted to 1-3 × 10<sup>4</sup> colony-forming units (CFU)/mL with Sabouraud dextrose broth. Then 100 μL of inoculum was added to each tube (final volume 1 mL and final inoculum level 1-3 × 10<sup>3</sup> CFU/mL) and incubated at 30°C for 3-6 days (until clear growth was visible in the control) depending on the species. Growth was read visually after the incubation and MIC was determined as the lowest drug concentration at which no visible growth was observed.

For determining fungicidal activity, contents from tubes with no visible growth were subcultured on Sabouraud dextrose agar plates (in triplicates). After mixing the contents well, 50 μL samples were drawn from each tube and then spread over the surface of agar by tilting the plate. The plates were incubated at 30°C for 7 days. Minimum fungicidal concentration (MFC) was determined as the lowest concentration of the compound with which all subcultures were negative.

### Protease inhibition assay

Trypsin was the enzyme used for the study. Hundred microliter of trypsin (1 mg/mL of 1 mM HCl) was added to 50 μL of different concentrations of zerumbone (10, 50, 100, 500, 1000 and 2000 μg) in DMSO and made up to 1 mL with Tris-HCl buffer (50 mM (pH 7.8) containing 1 mM CaCl<sub>2</sub>). For test blanks, 100 μL of 1 mM HCl was taken instead of trypsin. For control, 50 μL DMSO was taken instead of test. All tubes were incubated at room temperature for 10 min. Then 1 mL of BSA (4 g% in Tris-HCl buffer) was added to all tubes and digestion was carried out at 37°C in a water bath for 20 min. Three millilitre of 5% trichloro acetic acid (TCA) was added to stop the reaction, centrifuged at 2500 rpm and absorbance of supernatant was read at 280 nm. For control blanks, 100 μL of trypsin and 50 μL DMSO, made up to 1 mL with buffer, was mixed with 1 mL of BSA. To this, 3 mL of 5% TCA was added immediately and centrifuged. Buffer was used to adjust auto zero.<sup>[12]</sup>

The percentage of inhibition was calculated as,

$$\frac{(\text{absorbance of control} - \text{absorbance of test})}{\text{absorbance of control}} \times 100$$

Diclofenac sodium (Sigma) was used as the reference drug (10, 50, 100, 500, 1000 and 2000 μg). IC<sub>50</sub> values were calculated by plotting inhibition percentage against concentration of test materials. Control and tests were done in triplicate and the results are expressed as mean ± SD, where *n* = 3 (calculated using Microsoft Office Excel 2007).

## Determination of *in vitro* cytotoxic effect on cultured Chang liver cell lines–MTT assay

Chang liver cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and grown to reach confluency at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere in a CO<sub>2</sub> incubator (NBS, Eppendorf, Germany). The cells were trypsinized (0.025% trypsin in phosphate-buffered saline (PBS)/0.5 mM EDTA solution) and were placed into 96-well tissue culture plates at a density of 1 × 10<sup>5</sup> cells per well and incubated (24 h) to assure 70-80% confluency. Test materials were added to grown cells at a final concentration of 6.25, 12.5, 25, 50, 100 and 200 µg/L and incubated for 24 h.

Cells were washed with 1× PBS and fresh medium was added along with 30 µL of MTT solution (5 mg/mL dissolved in PBS). Then incubated at 37°C for three hours and MTT was removed by washing with 1 × PBS. Two hundred microliter of DMSO was added to the culture and incubated at room temperature for 30 min to dissolve the formazan crystals formed. Cell debris was precipitated by centrifugation and optical density was read at 570 nm using DMSO as blank in a microplate reader (ELISACAN, ERBA).<sup>[13,14]</sup> Percentage viability was calculated as follows:

$$\text{Percentage viability} = (\text{OD of test}/\text{OD of control}) \times 100$$

Control and tests were done in triplicate and the results are expressed as mean ± SD, where  $n = 3$  (calculated using Microsoft Office Excel 2007). Doxorubicin (anticancer drug) was used as the reference drug (concentration range tested was 0.1-0.6 µg/mL). IC<sub>50</sub> value was calculated by plotting percentage viability of cells against concentration of test materials.

## RESULTS

### Isolation and purification of zerumbone

HPLC analysis of the isolated crystal showed single peak at retention time 7.967. This confirmed purity of the isolated crystal. Peaks in IR spectrum were in agreement with the structure of zerumbone. IR (ATR-FTIR)  $\nu_{\text{max}}$  (cm<sup>-1</sup>): 3030, 2923, 2961, 1728, 1652, 1455, 1385, 1263, 965, 827. Features of <sup>13</sup>C NMR and <sup>1</sup>H NMR spectra of purified compound coincided with the structure of zerumbone. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>), <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz): data shown in Table 1.

The mass spectrum showed the presence of a molecular ion at  $m/z$  219.1755. Since the spectrum was taken under positive ionisation mode (M+H)<sup>+</sup>, the molecular weight of the isolated compound could be calculated as 218.1755, which corresponded to that of zerumbone. Also, elemental composition analysis of the mass spectrum confirmed the molecular formula as C<sub>15</sub>H<sub>25</sub>O (M+H)<sup>+</sup>. Hence, the molecular formula of the isolated compound is C<sub>15</sub>H<sub>22</sub>O, which is that of zerumbone. Thus, FT-IR, NMR, and LC-MS analyses confirmed that the isolated compound is zerumbone.

### Antidermatophytic assay

MIC and MFC of zerumbone and griseofulvin for the tested dermatophytes are shown in Table 2. Zerumbone was fungicidal towards all the tested dermatophytes, but MFC values were higher than the MIC values. The tested organisms were sensitive to the reference drug griseofulvin. MIC values of zerumbone were higher when compared with griseofulvin, but MFC values were the same.

### Protease-inhibiting assay

Zerumbone exhibited good protease (trypsin) inhibiting activity [Figure 1]. IC<sub>50</sub> of trypsin activity for zerumbone was 120 µg and that of diclofenac sodium was 15 µg.

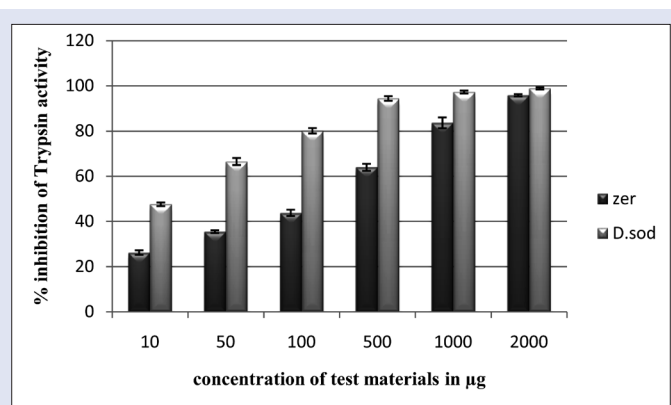
**Table 1:** <sup>1</sup>H (400MHz) and <sup>13</sup>C (75 MHz) NMR data of purified component in CDCl<sub>3</sub> (δ in ppm, J in Hz)

Position	δ c	δ H
1	204.31	
2	160.70	
3	148.76	6.03 (m, 1H)
4	24.42	2.10 – 2.50 (m, 4H)
5	39.48	2.10 – 2.50 (m, 4H)
6	138	
7	136.29	5.25 (m, 1H)
8	42.45	1.90 (d, 2H)
9	37.88	
10	127.20	5.98 (d, J=16.4 Hz, 1H)
11	125.03	5.86 (d, J=16.4 Hz, 1H)
12	15.22	1.54 (s, 3H)
13	11.79	1.80 (s, 3H)
14	24.20	1.07 or 1.22 (s, 3H)
15	29.44	1.07 or 1.22 (s, 3H)

**Table 2:** MIC and MFC of zerumbone and griseofulvin against *E. floccosum*, *M. canis*, *M. gypseum* and *T. rubrum*

Compound		Organism			
		<i>E.floccosum</i>	<i>M.canis</i>	<i>M.gypseum</i>	<i>T.rubrum</i>
Zerumbone	MIC*	8	8	16	16
	MFC*	64	64	128	128
Griseofulvin	MIC*	2	4	2	4
	MFC*	64	64	128	128

\*MIC and MFC expressed in mg/L.



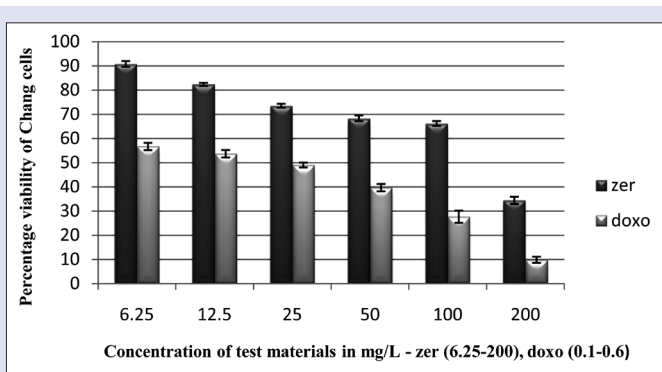
**Figure 1:** Percentage inhibition of trypsin activity by zerumbone and diclofenac sodium. zer, zerumbone; D. sod, diclofenac sodium

## Determination of *in vitro* cytotoxic effect on cultured Chang liver cell lines–MTT assay

Effect of zerumbone and doxorubicin on the percentage viability of Chang liver cells at different concentrations is depicted in Figure 2. The percentage viability of Chang liver cells in the presence of zerumbone was very high compared with doxorubicin. IC<sub>50</sub> of zerumbone against the viability of Chang liver cells was 150 µg and that of doxorubicin was 0.31 µg. This showed low toxicity of zerumbone towards Chang liver cells.

## DISCUSSION

The incidence of dermatophytoses has risen dramatically in recent years due to expanding population of individuals with impaired immunity, such as AIDS



**Figure 2:** Percentage viability of Chang liver cells in the presence of zerumbone and doxorubicin. zer, zerumbone; doxo, doxorubicin

patients, organ transplant recipients, cancer patients, and so on. Griseofulvin, azole derivatives, and allylamines are the major antifungal agents currently used in the treatment of dermatophytoses. Lack of fungicidal activity, drug-drug interaction, hepatotoxicity, gastrointestinal disturbances, and anemia are the major drawbacks of these currently used antidermatophytic agents.<sup>[15-18]</sup> Also, emergence of drug resistance can lead to treatment failure using these conventional agents.<sup>[19,20]</sup> This justifies the search for newer antifungal drugs. Zerumbone is a sesquiterpene present abundantly in the rhizome of *Z. zerumbet* and an emerging anticancer agent.<sup>[4,5]</sup> In the present study, zerumbone exhibited fungicidal activity toward all the dermatophytes tested.

In dermatophytic infections, the fungus causes only minimum damage to the skin directly. Most of the pathology is caused by the reaction of the host to the fungus and its metabolic products. The recovery from dermatophytic infections depends on fungal growth restriction as well as resolution of inflammatory pathology.<sup>[21]</sup> Hence, use of topical agents with antifungal and anti-inflammatory activity is the emerging trend in the treatment of dermatophytoses. Zerumbone possesses very good anti-inflammatory effect, which is evidenced from its cyclooxygenase (COX), lipoxygenase (LOX), myeloperoxidase (MPO), and nitric oxide synthase inhibiting activities (unpublished results) and trypsin-inhibiting activity. Trypsin inhibition assay is a commonly used method for investigating anti-inflammatory property of materials.<sup>[22]</sup> Trypsin is a serine protease. In addition to the role in protein degradation, proteases (proteinases) are involved in different physiological and pathophysiological processes. Some proteases signal to cells by activating a family of G-protein coupled receptors known as proteinase-activated receptors (PARs).<sup>[23]</sup> Four PARs have been identified till date namely, PAR-1, PAR-2, PAR-3 and PAR-4. PAR-1, PAR-3 and PAR-4 are activated by thrombin and PAR-2 by trypsin or tryptase.<sup>[24]</sup> PAR-2 plays a substantial role in different physiological and pathophysiological processes in various organ systems. Acute activation of PAR-2 functions to help in physiological processes, whereas hyperactivation is seen in inflammation.<sup>[25]</sup> PAR-2 is abundantly expressed in the skin by almost all cell types, particularly keratinocytes. PAR-2 is a main sensor for exogenous proteases from different allergens.<sup>[26]</sup> It has been reported that serine protease secreted by the dermatophytes causes itching through the activation of PAR-2 receptors.<sup>[2]</sup> Abundant expression of PAR-2 in keratinocytes substantiates this report. Since trypsin is a serine protease, the materials having trypsin-inhibiting activity might be able to inhibit serine proteases secreted by dermatophytes and will help to provide relief from itching. Geophilic and zoophilic dermatophytes cause severe inflammatory reactions because of the delayed hypersensitivity responses to fungal proteases.<sup>[19]</sup> If the antifungal agent is having protease-inhibiting activity, it will help in restricting such kinds of inflammatory responses.

Cyclooxygenases are enzymes that catalyse the formation of prostaglandins from arachidonic acid. Prostaglandins are lipid molecules which have significant role in eliciting the inflammatory response. 5-Lipoxygenase synthesises leukotrienes from arachidonic acid which also are lipid molecules that act as mediators of inflammation. Dermatophytes are capable of both trans-species and de-novo production of leukotrienes and prostaglandins during infection. Enhanced prostaglandin production during infection might be an important factor promoting fungal colonisation and chronic infection.<sup>[27]</sup> Hence, COX and 5-LOX inhibiting ability of zerumbone may also support fungal growth restriction by the compound, in addition to the anti-inflammatory efficacy.

Treatment with oral antifungal agent is usually required in *Tinea unguium* (nail ring worm) and in extensive dermatophytosis. Hepatotoxicity is the major side effect associated with currently used antifungal drugs. *In-vitro* studies using cell lines represent first phase in the evaluation of the toxicity of drugs. In the MTT assay using Chang liver cell line, the cytotoxicity of zerumbone was found to be very low compared with the reference anti-cancer drug doxorubicin. IC<sub>50</sub> values were 150 and 0.31 µg, respectively, for zerumbone and doxorubicin. MIC of zerumbone was 8 mg/L against *E. floccosum* and *M. canis* and 16 mg/L for *M. gypseum* and *T. rubrum*. MFC of zerumbone was 64 mg/L against *E. floccosum* and *M. canis* and 128 mg/L for *M. gypseum* and *T. rubrum*. At oral doses of 100 and 200 mg/kg, zerumbone has been reported to possess no acute toxicity.<sup>[28,29]</sup> Thus, from the MTT assay and earlier reports on acute toxicity study, it can be concluded that zerumbone might not be hepatotoxic at the concentrations required for use as antidermatophytic agent.

## CONCLUSION

Treatment with topical preparations is the first choice in dermatophytosis therapy. Agents with antidermatophytic as well as anti-inflammatory activity are preferred for topical use, since they can provide rapid symptomatic relief. Protease-inhibiting ability of the agents will help to relieve the patient from itching associated with dermatophytosis. Many of the currently used agents are fungistatic only and hence have to be applied for a long period of time. In the present study, zerumbone exhibited fungicidal activity against the dermatophytes tested. Hence, it is concluded that zerumbone could be recommended as a novel product for topical use in dermatophytoses therapy because of its combined fungicidal, anti-inflammatory and protease-inhibiting properties, after further clinical studies. From the MIC and MFC values, MTT assay and earlier studies on acute toxicity, it can be suggested that zerumbone might not be hepatotoxic in oral use at concentration required for antidermatophytic activity. The fact that zerumbone is a natural compound from the rhizome of *Z. zerumbet* which is a traditional food-flavouring agent supports this anticipation. However, studies on toxicity using animal models and determination of optimal concentration are necessary before any possible oral application of the compound.

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## Conflicts of interest

There are no conflicts of interest.

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