EVALUATION OF THE CONCENTRATIONS OF *CURCUMA LONGA* (TURMERIC) ESSENTIAL OIL TO CAUSE FUNGISTASIS AND INHIBIT MELANOGENESIS IN THE FUNGUS *NEOSCYTALIDIUM DIMIDIATUM*

Kunyanat Krongboon[1], Ariya Chindamporn[2], Sirida Youngchim[3], Navaporn Worasilchai[4,5], Patcharin Thammasit[3], Siriporn Chongkae[3] and Pornchai Sithisarankul[1]

[1]Department of Preventive and Social Medicine, [2]Department of Microbiology, Faculty of Medicine, Chulalongkorn University; [3]Department of Microbiology Faculty of Medicine, Chiang Mai University; [4]Department of Transfusion Medicine and Clinical Microbiology, [5]Research Unit of Medical Mycology Diagnosis, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand

Abstract. *Neoscytalidium dimidiatum*, a cause of onychomycosis and tinea pedis, produces melanin, a virulence factor. In this study, we aimed to do 2 things: 1) determine the concentration of *Curcuma longa* (turmeric) essential oil to be fungistatic against *N. dimidiatum in vitro* and 2) determine the ability of *Curcuma longa* (turmeric) essential oil to prevent melanogenesis (a virulence factor) by *N. dimidiatum in vitro* in order to determine its potential use as an antifungal agent. The testing was done using 10 clinical isolates of *N. dimidiatum*. The concentrations of turmeric essential oil used for this study ranged from 2% to 20%. Sensitivity testing was done using an agar dilution method with a 7-day incubation period to determine the minimal inhibitory dilution. The ability to inhibit melanogenesis was evaluated by comparing the amount of melanin between untreated isolates and isolates treated with either 4% or 10% turmeric essential oil. Melanin detection was accomplished using melanin-specific monoclonal antibody (Mab) 8D6 and measuring the immunofluorescence using an immunofluorescent microscope at 1,000x magnification. The concentration of turmeric essential oil that significantly inhibited *N. dimidiatum* growth was the 20% solution. The 4% and 10% turmeric essential oil preparation treated isolates of *N. dimidiatum* gave significantly lower (1.592 (p=0.001) and 1.705 (p=0.003)) mean fluorescent intensities than the untreated isolate (7.575). In summary, 20% turmeric essential oil significantly inhibited *N. dimidiatum* growth and both 4% and 10% turmeric essential oil samples inhibited melanogenesis. We conclude turmeric essential oil has therapeutic potential for use against infections caused by *N. dimidiatum*.

Further studies are needed to determine what the antifungal mechanism is and if it can be used *in vivo* to treatment these infections.

Keywords: *Curcuma longa* (turmeric) essential oil, fungistatic, melanogenesis, *Neoscytalidium dimidiatum*, onychomycosis

Correspondence: Pornchai Sithisarankul, Department of Preventive and Social Medicine, Faculty of Medicine, Chulalongkorn University, 1873 Rama 4 Road, Pathumwan District, Bangkok 10330, Thailand
Tel: +66 (06) 3442 9653, +66 (08) 9886 7824
E-mail: k_d5058@hotmail.com; psithisarankul@gmail.com

INTRODUCTION

Onychomycosis is a common nail infection worldwide (Gupta *et al*, 2020). About 60-70% of onychomycosis cases are caused by *Trichophyton rubrum* or *Trichophyton mentagrophytes* and the rest are primarily caused by *Epidermophyton floccosum* or some other dermatophyte (Augustin *et al*, 2013; Ghannoum *et al*, 2000). Non-dermatophyte molds (NDMs), such as *Scopulariopsis brevicaulis*, *Fusarium* spp, *Aspergillus* spp, *Acremonium* spp, *Neoscytalidium dimidiatum*, and yeasts, particularly *Candida* spp, have also been found to cause onychomycosis, particularly in warmer regions (Gupta *et al*, 2020; Di Chiacchio *et al*, 2013; Ghannoum *et al*, 2000; Gupta *et al*, 2012; Kaur *et al*, 2008). The prevalence of onychomycosis and their causative fungi varies by region. A study from Thailand reported onychomycosis was caused by non-dermatophyte molds in 51.6%, dermatophytes in 36.3% and *Candida* spp in 6.0% with *N. dimidiatum* being the most common non-dermatophyte mold and *Trichophyton rubrum* and *Trichophyton mentagrophytes* being the most common dermatophytes (Ungpakorn *et al*, 2004). Mixed infections, with both dermatophytes and non-dermatophyte molds have been reported to cause 3-11% of onychomycosis cases (Moreno and Arenas, 2010; Salakshna *et al*, 2018; Vander Straten *et al*, 2002). *N. dimidiatum* is a dematiaceous black fungus characterized by melanin or melanin-like pigment in the walls of its spores and hyphae (Ungpakorn *et al*, 2004).

Melanin contributes to the pathogenicity of *N. dimidiatum* (Nosanchuk *et al*, 1998) and protects it from harsh environmental conditions (Polak, 1990). Fungi that produce melanin can better withstand high temperatures and ultraviolet, gamma and x-ray radiation than those which do not produce melanin (Bell and Wheeler, 1986; Mirchink *et al*, 1972; Wheeler and Bell, 1988). The presence of melanin reduces susceptibility to antifungal agents and inhibition of melanin production can increase susceptibility to antifungal agents (Heidrich *et al*, 2021). Some mammalian immune systems detect and bind to melanin (Smith and Casadevall, 2019). *Neoscytalidium* spp produce melanin and are resistant to the majority of topical and systemic antifungals, such as griseofulvin, ketoconazole, fluconazole, itraconazole and terbinafine (Machouart *et al*, 2013).

Curcuma longa (turmeric) essential oil, containing turmerone, atlantone, zingiberone and some curcuminoids, has been reported to have antifungal properties (Apisariyakul *et al*, 1995; Pothitirat and Gritsanapan, 2006). *C. longa* (turmeric) essential oil may also inhibit melanin production (Moghadamtousi *et al*, 2014). In this study, we aimed to do 2 things: 1) determine the concentration of *C. longa* (turmeric) essential oil to be fungistatic against *N. dimidiatum in vitro* and 2) determine the ability of *C. longa* (turmeric) essential oil to prevent melanogenesis (a virulence factor) by *N. dimidiatum in vitro* in order to determine its potential use as an antifungal agent.

MATERIALS AND METHODS

Obtaining clinical isolates

In order to conduct this study, we obtained 10 clinical isolates of *Neoscytalidium dimidiatum* from the Institute of Dermatology, Bangkok, Thailand: 3 from otherwise healthy subjects, 5 from subjects with metabolic syndrome and 2 from subjects with chronic allergies.

Obtaining the turmeric essential oil

The turmeric essential oil concentrations used for this study ranged from 2% to 20%, and were obtained from the Thai-China Flavors and Fragrances Industry Co Ltd, Phra Nakhon Si Ayutthaya Province, Thailand.

The manufacturer of the tested turmeric essential oil used gas chromatography-mass spectrometry (GC-MS) to evaluate the 100% concentration of the turmeric essential oil and found it consisted of 42.7% turmerone.

Identification and culture of *Neoscytalidium dimidiatum*

N. dimidiatum isolates were identified using the internal transcribed spacer-polymerase chain reaction (ITS-PCR) method by performing DNA extraction of 2 mg of fungal sample using the GenUp gDNA extraction kit (Sigma-Aldrich, Darmstadt, Germany). The extracted DNA was stored at -20°C until used. ITS-PCR testing was performed following the method described by White (1990). *N. dimidiatum* was identified by ITS-PCR using the ITS-1 forward primer (5′-TCCGTAGGTGAACCTGCGG-3′) and the ITS-2 reverse primer (5′-GCTGCGTTCTTCATCGATGC-3′). The PCR product was placed on 2% agarose gel containing 0.5× Tris-borate-EDTA (TBE) and gel electrophoresis was conducted at 100 volts. The DNA bands were stained with ethidium bromide 1 µg/ml for 5 minutes. The bands were then observed under ultraviolet light using the Syngene G Box Gel documentation system (Syngene, Frederick, MD). The bands were compared with a low molecular weight DNA ladder (New England Biolabs, Ipswich, MA). The ITS1 DNA sequences were analyzed using DNA sequencing (3500 Genetic analyzer, Applied Biosystems, Foster City, CA). The DNA sequences were compared with the GENBANK database using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the fungal species; a similarity score of at least 95% was used for identification (Jang *et al*, 2012). The fungus was cultured on potato dextrose agar medium (Biomedia, Nonthaburi Province, Thailand) at a temperature of 28°C for one week.

Antifungal properties of turmeric essential oil

An agar dilution method was used to determine the percentage of turmeric essential oil needed to inhibit *N. dimidiatum* growth following the modified Clinical and Laboratory Standards Institute (CLSI) recommendation M07 (CLSI, 2018). A conidia suspension was prepared to obtain a final fungal concentration of 0.4 x106 CFU/ml using a hemacytometer (American Optical, Southbridge, MA). A 100 µl inoculum of the prepared suspension was cultured on multiple Muller Hinton agar (Sigma-Aldrich, Darmstadt, Germany) plates and each plate was treated with turmeric essential oil at with one of the following concentrations: 2%, 3%, 4%, 5%, 10% and 20% and then each plate was incubated at 35°C for 48 hours. The minimal inhibitory

concentration was the concentration at which there was no fungal growth. The color of the colonies in the untreated and each of the treated plates was observed 7 days after onset of treatment.

Determination of melanin quantity using melanin-specific monoclonal antibody 8D6 immunofluorescence

Melanin production was determined using immunofluorescent (IF) analysis using a method described previously (Chongkae *et al*, 2019). Briefly, isolates of *N. dimidiatum* were grown on Muller Hinton agar for 14 days and the colonies obtained were divided into 4 groups: untreated, exposed to 4% turmeric essential oil, exposed to 10% turmeric essential oil and exposed to 10 µg Itraconazole (Sigma-Aldrich, Darmstadt, Germany), a medication commonly used to treat onychomycosis. After treatment, each specimen was washed with 95% ethanol and PBS for 1 minute and this was repeated for a total of 3 times. The cell walls of the specimens were then enzymatically digested using Novozymes (lysing enzymes from Trichoderma harzianum) (Sigma-Aldrich, Darmstadt, Germany) at a concentration of 10 mg/ml, followed by incubation at 30°C for 24 hours. Then each specimen was washed with PBS 3 times, stained with monoclonal antibody 8D6 against melanin at a concentration of 20 µg/ml (Youngchim *et al*, 2004). Each specimen was then incubated at 37°C for 1 hour and then washed with PBS 3 times and stained with Alexa Fluor-488-conjugated goat anti-mouse IgM antibody (Sigma-Aldrich, Darmstadt, Germany) at a dilution of 1:500. The intensity of fluorescence was measured using a Nikon ECLIPSE 50 I fluorescence microscope (Nikon, Tokyo City, Japan). Quality control was testing using *N. dimidiatum* as a positive control and *N. hyalinum* as a negative control.

Statistical analysis

Means and standard deviations were used to specify the intensity of IF and to calculate the percent inhibition of fungal growth calculated as the difference between the initial amount of fungus in the inoculum and the amount of fungus after 7 days incubation, divided by the initial amount of fungus in the inoculum multiplied by 100.

The paired t-test was used to compare the mean IF between the untreated group and the groups treated with 4% turmeric essential oil, 10% turmeric essential oil or 10 µg itraconazole.

Ethical consideration

The study was approved by the ethics committee of Institutional Review Board of Dermatology Bangkok, Thailand (Document No. IRB 035/2564).

RESULTS

Inhibitory effect of *Curcuma longa* (turmeric) essential oil

Twenty-percent turmeric essential oil gave 100% inhibition of *Neoscytalidium dimidiatum* growth (Table 1).

Inhibition of melanin

IF intensity testing to detect the amount of melanin in the untreated specimens compared with the specimens treated with 4% turmeric essential oil, 10% turmeric essential oil and 10 µg itraconazole revealed intensities of 7.575, 5.983 (p=0.001), 5.867 (0.003) and 6.417 (p=0.101) arbitrary units, respectively (Table 2, Fig 1, Figs 2E-2L).

The color of the untreated *N. dimidiatum* colonies and the colonies treated with 10 µg itraconazole were black and the colors of the colonies treated with 4% turmeric essential oil and 10% turmeric essential oil were creamy white (Figs 2A-2D).

Table 1

Percent inhibition of *Neoscytalidium dimidiatum* by various concentrations of turmeric essential oil

% Turmeric essential oil	Growth at 7 days, CFU/ml (n = 10)	% Inhibition
2	0.4×10^5	0.001
3	0.4×10^5	0.001
4	0.4×10^5	0.001
5	0.4×10^4	0.01
10	0.4×10^3	0.1
20	0	100

CFU: Colony Forming Unit, ml: milliliters

Table 2

Comparison of immunofluorescent intensity among treated and untreated samples

Comparison groups (n = 50)	Immunofluorescent intensity Mean ± SD		Mean differences	SEM	95% CI for the difference	*p*-value
	Untreated, AU	Treated, AU				
Comparison of untreated with 4% turmeric essential oil treated results	7.575 ± 2.914	5.983 ± 2.155	1.592	0.472	0.644-2.540	0.001
Comparison of untreated with 10% turmeric essential oil treated results	7.575 ± 2.914	5.870 ± 2.330	1.705	0.538	0.624-2.786	0.003
Comparison of untreated with 10 μg itraconazole treated results	7.575 ± 2.914	6.417 ± 4.085	1.158	0.693	0.234-2.550	0.101

AU: arbitrary units; CI: confidence interval; SD: standard deviation; SEM: standard error of the mean

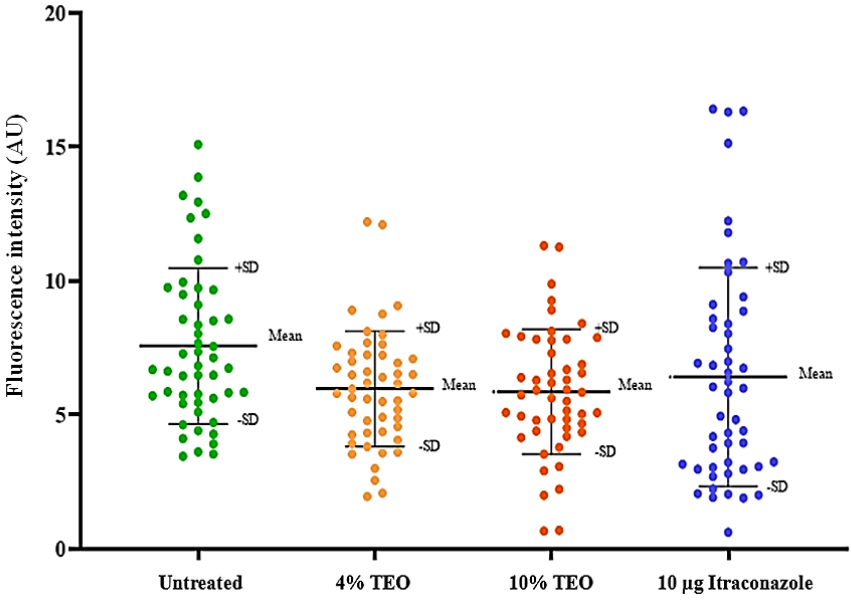

Fig 1 - Fluorescence intensity of *Neoscytalidium dimidiatum* stained with melanin-specific monoclonal antibody 8D6

AU: arbitrary units; SD: standard deviation; TEO: turmeric essential oil; µg: micrograms

DISCUSSION

In our study, turmeric essential oil inhibited *Neoscytalidium dimidiatum* growth similar to another study that reported turmeric acid essential oil inhibited *Sporothrix schenckii, Exophiala jeanselmei, Fonsecaea pedrosoi* and *Scedosporium apiospermum* growth (Apisariyakul *et al*, 1995). The mechanism of this inhibition is thought to be due to turmerones (Kumar *et al*, 2016), specifically sesquiterpenoid α- and β-turmerone, major sesquiterpenes of the bisabolane class (Awasthi and Dixit, 2009; Zhang and Kitts, 2021). These unsaturated ketones interact with the plasma membrane, modifying fatty acid composition and changing cell membrane permeability (Kumar *et al*, 2016).

In our study, 4% and 10% turmeric essential oil resulted in significantly lower *N. dimidiatum* melanin levels than the untreated group but the 10 µg itraconazole treated group

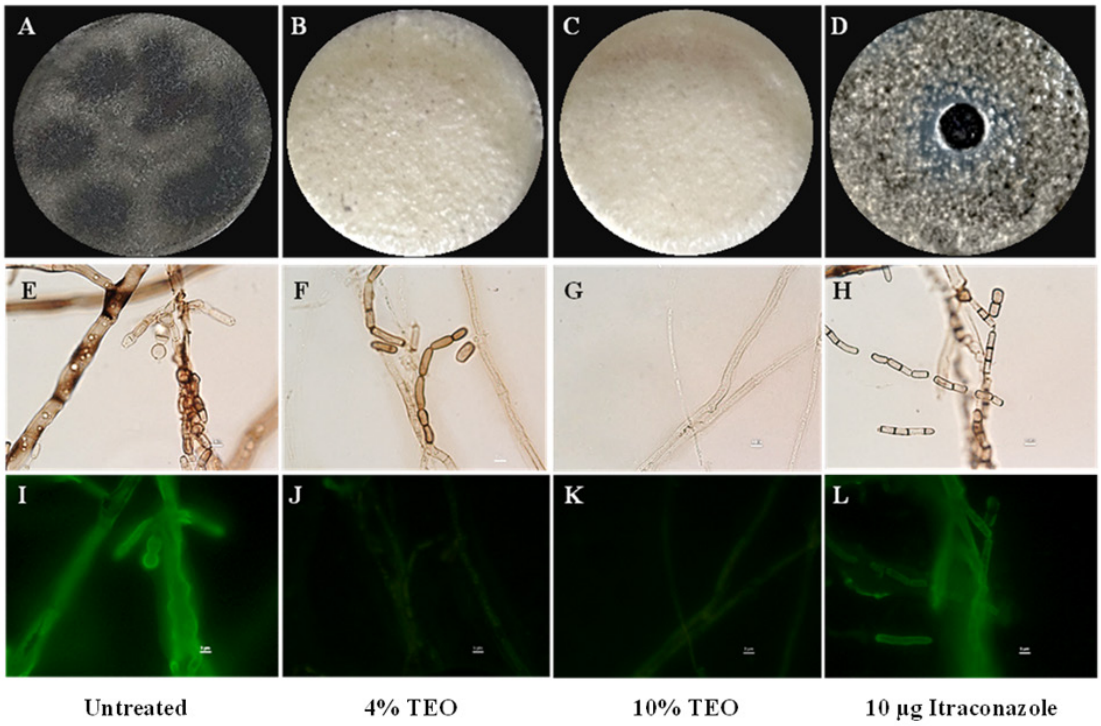

Fig 2 - Effects of turmeric essential oil and itraconazole on *Neoscytalidium dimidiatum*: culture colony, microscopy, and immunofluorescent microscopy analysis

Photograph of *Neoscytalidium dimidiatum* culture - A: untreated; B: treated with 4% turmeric essential oil; C: treated with 10% turmeric essential oil; D: treated with 10 microgram (µg) itraconazole

Microscopy of *Neoscytalidium dimidiatum* (1000x) - E: untreated; F: treated with 4% turmeric essential oil; G: treated with 10% turmeric essential oil; H: treated with 10 µg itraconazole.

Immunofluorescent microscopy of *Neoscytalidium dimidiatum* (1000x) stained with melanin-specific monoclonal antibody 8D6 - I: untreated; J: treated with 4% turmeric essential oil; K: treated with 10% turmeric essential oil; L: treated with 10 µg itraconazole.

Scale bar = 5 micrometer (µm)

TEO: turmeric essential oil; µg: micrograms

did not. Itraconazole is commonly prescribed to treat onychomycosis. This suggests several possibilities: our study design may not accurately reflect *in vivo* treatment, our specimens may not represent the usual causes of onychomycosis, itraconazole may not be the best treatment for onychomycosis caused by *N. dimidiatum*; *N. dimidiatum* may not be a common cause of onychomycosis in our study population or turmeric essential oil may be a potentially better treatment for onychomycosis caused by *N. dimidiatum*. *N. dimidiatum* has been reported to have resistance to a variety of topical and systemic antifungal medications, including griseofulvin, ketoconazole, fluconazole, itraconazole and terbinafine (Machouart *et al*, 2013).

A limitation of our study was the use of the micro-broth dilution method to determine the minimum inhibitory concentration (MIC) of the essential oil. This method we used is common but not standard practice (Castilho *et al*, 2015). The reason for this to not be standard practice is the challenge in detecting the results through observation. This is attributed to the lipophilic and insoluble properties of essential oil requiring some adjustments in the technique (Romano *et al*, 2009). To address this, we modified the agar dilution method following CLSI M07 guidelines (CLSI, 2018).

As far as we know, this is the first study to evaluate the fungistatic properties of turmeric essential oil against *N. dimidiatum* and the first to study the effects of this essential oil on melanogenesis. Our results can inform further efforts to evaluate the potential *in vivo* antifungal benefits of turmeric essential oil against *N. dimidiatum*. Our results also suggest a potential mechanism of targeting melanin in order to treat fungal infections caused by *N. dimidiatum*.

In summary, 20% turmeric essential oil significantly inhibited *Neoscytalidium dimidiatum* growth and both 4 and 10% turmeric essential oil samples inhibited melanogenesis, resulting in a change in the culture colony color suggesting loss of melanin. We conclude turmeric essential oil has therapeutic potential for use against infections caused by *N. dimidiatum*. Further studies are needed to determine what the antifungal mechanism is and if it can be used *in vivo* to treatment these infections.

ACKNOWLEDGEMENTS

The molecular probes used for this study were kindly provided by

Assoc Prof Dr Sirida Youngchim, Department of Microbiology, Faculty of Medicine, Chiang Mai University.

This study was funded by the Ratchadapiseksompotch Research Fund (GA 65-11), a Faculty of Medicine, Chulalongkorn University and Department of Medical Service Fund.

REFERENCES

Apisariyakul A, Vanittanakom N, Buddhasukh D. Antifungal activity of turmeric oil extracted from *Curcuma longa* (Zingiberaceae). *J Ethnopharmacol* 1995; 49(3): 163-9.

Augustin M, Radtke MA, Herberger K, Kornek T, Heigel H, Schaefer I. Prevalence and disease burden of hyperhidrosis in the adult population. *Dermatology* 2013; 227(1): 10-3.

Awasthi PK, Dixit SC. Chemical composition of *Curcuma longa* leaves and rhizome oil from the plains of Northern India. *J Young Pharm* 2009; 1(4): 312-6.

Bell AA, Wheeler MH. Biosynthesis and functions of fungal melanins. *Ann Rev Phytopathol* 1986; 24: 411-51.

Castilho AL, Caleffi-Ferracioli KR, Canezin PH, Dias Siqueira VL, de Lima Scodro RB, Cardoso RF. Detection of drug susceptibility in rapidly growing mycobacteria by resazurin broth microdilution assay. *J Microbiol Methods* 2015; 111: 119-21.

Chongkae S, Nosanchuk JD, Pruksaphon K, Laliam A, Pornsuwan S, Youngchim S. Production of melanin pigments in saprophytic fungi *in vitro* and during infection. *J Basic Microbiol* 2019; 59(11): 1092-104.

Clinical and Laboratory Standard Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically M07. 11th ed. Wayne: Clinical and Laboratory Standard Institute; 2018.

Di Chiacchio N, Suarez MV, Madeira CL, Loureiro WR. An observational and descriptive study of the epidemiology of and therapeutic approach to onychomycosis in dermatology offices in Brazil. *An Bras Dermatol* 2013; 88 (Suppl 1): 3-11.

Ghannoum MA, Hajjeh RA, Scher R, *et al*. A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns. *J Am Acad Dermatol* 2000; 43(4): 641-8.

Gupta AK, Drummond-Main C, Cooper EA, Brintnell W, Piraccini BM, Tosti A. Systematic review of nondermatophyte mold onychomycosis: diagnosis, clinical types, epidemiology, and treatment. *J Am Acad Dermatol* 2012; 66(3): 494-502.

Gupta AK, Stec N, Summerbell RC, *et al*. Onychomycosis: a review. *J Eur Acad Dermatol Venereol* 2020; 34(9): 1972-90.

Heidrich D, Pagani DM, Koehler A, Alves KO, Scroferneker ML. Effect of melanin biosynthesis inhibition on the antifungal susceptibility of chromoblastomycosis agents. *Antimicrob Agents Chemother* 2021; 65(8): e0054621.

Jang JH, Lee JH, Ki CS, Lee NY. Identification of clinical mold isolates by sequence analysis of the internal transcribed spacer region, ribosomal large-subunit D1/D2, and β-tubulin. *Ann Lab Med* 2012; 32(2): 126-32.

Kaur R, Kashyap B, Bhalla P. Onychomycosis-epidemiology diagnosis and management. *Indian J Med Microbiol* 2008; 26(2): 108-16.

Machouart M, Menir P, Helenon R, Quist D, Desbois N. Scytalidium and scytalidiosis: what's new in 2012? *J Mycol Med* 2013; 23(1): 40-6.

Mirchink TG, Kashkina GB, Abaturov IuD. Resistance of fungi with different pigments to - radiation. *Mikrobiologiia* 1972; 41(1): 83-6. [in Russian]

Moghadamtousi SZ, Kadir HA, Hassandarvish P, Tajik H, Abubakar S, Zandi K. A review on antibacterial, antiviral, and antifungal activity of curcumin. *Biomed Res Int* 2014; 2014: 186864.

Moreno G, Arenas R. Other fungi causing onychomycosis. *Clin Dermatol* 2010; 28(2): 160-3.

Kumar KN, Venkataramana M, Allen JA, Chandranayaka S, Murali HS, Batra HV. Role of *Curcuma longa* L. essential oil in controlling the growth and zearalenone production of *Fusarium graminearum*. *LWT Food Sci Technol* 2016; 69: 522-8.

Nosanchuk JD, Rosas AL, Casadevall A. The antibody response to fungal melanin in mice. *J Immunol* 1998; 160(12): 6026-31.

Polak A. Melanin as a virulence factor in pathogenic fungi. *Mycoses* 1990; 33(5): 215-24.

Pothitirat W, Gritsanapan W. Variation of bioactive components in *Curcuma longa* in Thailand. *Curr Sci* 2006; 91(10): 1397-400.

Romano CS, Abadi K, Repetto V, Vojnov AA, Moreno S. Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives. *Food Chem* 2009; 115(2): 456-61.

Salakshna N, Bunyaratavej S, Matthapan L, Lertrujiwanit K, Leeyaphan C. A cohort study of risk factors, clinical presentations, and outcomes for dermatophyte, nondermatophyte, and mixed toenail infections. *J Am Acad Dermatol* 2018; 79(6): 1145-6.

Smith DFQ, Casadevall A. The role of melanin in fungal pathogenesis for animal hosts.*Curr Top Microbiol Immunol* 2019; 422: 1-30.

Ungpakorn R, Lohaprathan S, Reangchainam S. Prevalence of foot diseases in outpatients attending the Institute of Dermatology, Bangkok, Thailand. *Clin Exp Dermatol* 2004; 29(1): 87-90.

Vander Straten MR, Balkis MM, Ghannoum MA. The role of nondermatophyte molds in onychomycosis: diagnosis and treatment. *Dermatol Ther* 2002; 15: 89-98.

Wheeler MH, Bell AA. Melanins and their importance in pathogenic fungi. *Curr Top Med Mycol* 1988; 2: 338-87.

White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego, CA: Academic Press; 1990. p. 315-22.

Youngchim S, Morris-Jones R, Hay RJ, Hamilton AJ. Production of melanin by *Aspergillus fumigatus*. *J Med Microbiol* 2004; 53 (Pt 3): 175-81.

Zhang HA, Kitts DD. Turmeric and its bioactive constituents trigger cell signaling mechanisms that protect against diabetes and cardiovascular diseases. *Mol Cell Biochem* 2021; 476(10): 3785-814.