HNUE JOURNAL OF SCIENCE DOI: 10.18173/2354-1059.2020-0051
Natural Sciences 2020, Volume 65, Issue 10, pp. 82-91
This paper is available online at http://stdb.hnue.edu.vn

GROWTH INHIBITORY ACTIVITIES OF THE RHIZOME CRUDE EXTRACT OF *Curcuma longa* ON THE HUMAN PATHOGENIC FUNGUS *Mucor circinelloides*

Nguyen Anh Phuong, Le Thi Tuyet Mai and Trieu Anh Trung
Faculty of Biology, Hanoi National University of Education

Abstract. Mucormycosis is an uncommon but life-threatening invasive fungal infection, mostly occurs in immunocompromised patients. Lacking the appropriate antifungal drugs is one of the reasons that lead to difficulties in the management of mucormycosis. *Curcuma longa* has been used traditionally and widely to treat various diseases, including fungal infections. In the search for novel antifungal compounds from natural resources, we evaluated the effect of rhizome crude extract of *C. longa* on *Mucor circinelloides* – a causal agent of mucormycosis. The results of screening, using broth dilution method and agar-well diffusion method, showed that the *C. longa* extract exhibited promising antifungal activity against the fungus *M. circinelloides*. In liquid medium, *C. longa* extract decreased the ability of spore germination and the speed of hyphae formation of *M. circinelloides* decreased by up to approximately 70% and 90%, respectively. Besides, in a solid medium, the crude extract presented similar activity with amphotericin B (400 µg/mL) in decreasing the growth of *M. circinelloides* by nearly 77%. Moreover, the extract of *C. longa* also likely to induce the yeast-like type of growth of the dimorphic *M. circinelloides* in the early stage. These results suggest the plant could be a potential source for further study on biochemical components and the mechanism of its antifungal activity.

Keywords: *Curcuma longa, Mucor circinelloides,* mucormycosis.

1. Introduction

Mucormycosis (previously called zygomycosis), refers to several different diseases caused by fungi in the order Mucorales, which is described as an infrequently but acute infection, with a difficult diagnosis, accelerated progress, and unsatisfactory treatment, leading to exceptionally high mortality rates [1]. The lethal infection generally afflicting immunocompromised patients as an opportunistic infection with risk factors include diabetes mellitus, neutropenia, HIV/AIDS, organ transplant, broad-spectrum antibiotic using, etc. [1-3]. Immunocompromised patients who suffered severe injuries that primarily

Received June 17, 2020. Revised October 16, 2020. Accepted October 23, 2020.
Contact Trieu Anh Trung, e-mail address: trungta@hnue.edu.vn

break the integrity of the cutaneous barrier are also able to be affected [3]. The mortality rate can approach more than 90%, while the overall all-cause mortality rate surpasses 50% [4]. Recent reports have shown that mucormycosis has emerged as the second most common invasive mold infection [5, 6].

Mucor circinelloides, one causal agent of mucormycosis, is a ubiquitous dimorphic fungal belong to Mucorales, which can be found in soil and decaying matter. To be described relating to the virulence, the pathogenic fungus exhibits either hyphae or yeast-like growth depending upon the level of O_2 in the culturing conditions [7-9]. The size and the shape of sporangiospores are also linked to the virulence of *M. circinelloides* [10].

In current practice, antifungal therapy is one of the essential elements in the treatment of mucormycosis. Nevertheless, one of the obstacles that lead to difficulties in the management of the fatal disease is the variability of susceptibility to the first-line antifungal agent (amphotericin B) and the resistance to most other conventional antifungal agents [2]. Moreover, the treatment with amphotericin B, especially in long-term periods, can lead to some unexpected side effects in patients [11]. Therefore, it is a question of developing innovative antifungal therapies.

Curcuma longa Linn. (Turmeric), which belongs to the Zingiberaceae family, is used widely and traditionally, fresh or dried, in food as a spice, in cosmetics, pharmaceutical [12], and also served as a food coloring and chemical indicator [13]. Extract and the essential oil from *C. longa* inhibit the growth of a variety of bacteria, parasites, and pathogenic fungi. The extracts of *C. longa* in different solvents as well as the *C. longa* essential oils demonstrated effective activities against numerous phytophagous fungi [14, 15]. *C. longa* extracts and distilled oil also showed potent antifungal activities to many pathogenic yeasts and molds [16-18]. Noticeably, ethanol crude extract of *C. longa* presents considerable activities against several species of Mucorales, including *Rhizopus oryzae* - the most causative organism of mucormycosis, along with other two *Mucor* species [19, 20]. The amazing antifungal abilities of *C. longa* suggest that the plant extract might contain promising compounds that could control *M. circinelloides.* Thus, in this study, the rhizome crude extract of *C. longa* had been screened for the potential of inhibition against the pathogenic fungal *M. circinelloides.*

2. Content

2.1. Materials

**** Plant materials***

The fresh rhizome of *C. longa* was kindly provided and identified by the Department of Botany, Faculty of Biology, Hanoi National University of Education.

**** Fungal strain***

The wild type for carotenogenesis, leucine-auxotrophic (*leuA*$^-$) R7B strain of *M. circinelloides* (kindly provided by University of Murcia, Spain), was used for testing the antifungal activities of the plant extracts. It is a mutant strain derived from the sequenced CBS277.29 strain by the Joint Genome Institute [21].

2.2. Methods

2.2.1. Preparation of plant extracts

Fresh material was collected and washed thoroughly, first with running tap water, and then with distilled water, oven-dried at 50 ^{o}C until completely dry. The dried plant was ground to a fine powder, sealed carefully, and stored at -20 ^{o}C until use.

The plant extract was prepared according to the previously described protocol by Nguyen *et al*. [22] with some modifications. The amount of 1 *g* dried plant material was extracted with 10 mL of methanol, shaken vigorously, then soaking at 50 ^{o}C for at least 24 hours. The liquid was filtered, then concentrated by extensive evaporating of the solvent using an air dryer at room temperature. The collected extract was dissolved properly in ethanol (1 mL per 1 g dried powder), then centrifuged (5000 rpm, 5 minutes). The supernatant was collected and adjusted to the final solution of 1 g/mL (plant dried weight equivalent, DWE) in concentration.

2.2.2. Fungal culture and the collection of vegetative spores

M. *circinelloides* was cultured and grown to collect the vegetative spores according to the protocols (Basic Protocol 1 and Basic Protocol 2) indicated by Vellanki *et al.* [23]. The growth and sporulation of *M. circinelloides* were proceeded on a solid medium, using YPG pH 4.5 (Yeast-Peptone-Glucose media) agar plates. Besides, the fungus was grown in a growth chamber with continuous light at 26 ^{o}C. The collection of fresh spores generated from mycelia on a solid medium resulted in the sporangial suspensions in sterile distilled water. The sporangial suspension concentration was estimated using a Neubauer cell-counting chamber, then maintained with periodic sub-culturing at 4°C.

2.2.3. Evaluate the antifungal activities of the plant extracts

The bioassay procedure to determine the antifungal activities of natural plant extracts, in comparison with the liposomal form of amphotericin B, was obtained by using two methods: broth dilution and agar-well diffusion.

**** Amphotericin B agent preparation***

The powder of amphotericin B for injection (AMPHOTRET, Bharat Serums and Vaccines Limited, India) was diluted with sterilized deionized water and adjust to the concentration of 1 mg/mL (stock solution) right before use.

**** Broth dilution method***

The assay was conducted based on the method of Clinical and Laboratory Standards Institute (CLSI) [24] with modifications. The fresh vegetative spores of the R7B strain were cultured to have a 20 mL volume of pH 4.5 YPG liquid medium in a sterile 250 mL Erlenmeyer flask with the sporangial concentration was adjusted to 10^6 CFU/mL. Then, the prepared plant extract was added with an appropriate volume to obtain a serial of different concentrations, ranging from 0.5 to 4.0 mg/mL in DWE, while in control groups, absolute ethanol and solution of amphotericin B were added with the same volume. Prepared Erlenmeyer flasks were kept overnight at 4 ^{o}C, followed by incubating at 26 ^{o}C with shaking at 200 rpm for 6 - 7 hours. The bioassay was carried out in duplicate.

The specimens taken each hour were observed and photographed by using a computer connected microscope system (ZEISS Axio Scope.A1 with Axiocam 105

Color) and ZEN 2.6 software. All snapshots were used for acquiring data utilizing the calculation to obtain the germinating spore ratios (GSRs) and the polarity indexes (PIs) [25] in the following formulas:

$$\text{GSR} = \frac{\text{Number of germinated spores}}{\text{Total number of spores}}; \quad \text{PI} = \frac{\text{Cell length}}{\text{Cell width}}$$

The total number of spores and the number of germinated spores were counted manually. The length and the width of the fungal hyphae were measured utilizing ImageJ software (n = 50).

**** Agar-well diffusion method***

The experiment was designed as described by Andrew *et al.* [26] with some modifications. Plates for the tests were prepared by dispensing 30 mL of YPG (pH 4.5) sterile solid medium into Ø100 mm sterile Petri disks. Then 5×10^4 fungal spores of test strain were inoculated to the plate and uniformly spread out over the entire surface of the agar medium.

Cylindrical plugs having a diameter of 10.0 mm were removed from the solidified agar plates to create an aseptic well. Next, 200 µL of different diluted solutions of plant extracts were introduced to the wells to acquire a serial of concentrations, ranging from 50 to 400 mg/mL in DWE. Absolute ethanol and solution of amphotericin B 400 µg/mL were run as the negative and positive control groups for the tests. Plates prepared as described were kept at 4 ^{o}C overnight for the complete diffusion before incubating at 26 ^{o}C in the dark for 120 hours, and the diameters of the growth inhibition area (in millimeters, includes the good diameter) were measured every 24 hours. Morphology of fungal colonies and mycelia also were observed. The bioassay was performed in triplicate.

For each assay, the percentage inhibition of diameter growth (PIDGs) [27] and the relative inhibition zone diameter (RIZDs) [28] after 120 hours, in comparison with those of the negative and positive control groups, respectively, also were calculated according to the following equations.

$$\text{PIDG (\%)} = \frac{\text{Diameter of sample} - \text{Diameter of negative control}}{\text{Diameter of negative control}} \times 100$$

$$\text{RIZD (\%)} = \frac{\text{Diameter of sample} - \text{Diameter of negative control}}{\text{Diameter of positive control} - \text{Diameter of negative control}} \times 100.$$

2.2.4. Data analysis

Obtained data were kept in Microsoft Excel and analyzed using one-way analysis of variance (ANOVA) with significant $\alpha = 0.05$. Sample data that had *p*-values were less than α are considered to have statistical differences.

2.3. Results and discussions

2.3.1. Effects of the *C. longa* extract on the growth of *M. circinelloides* during the early stage

Since the ability of spore germination and the hyphae formation have been found to associate with the virulence of *M. circinelloides*, it is important to evaluate the effects of experimental plant extract on these features of the pathogenic fungus. The dilution method as described was used to assess those aspects in the early growth stage, in which the vegetative spores germinate and develop hyphae (from 0 to 12 hours of culturing) [29]. Data about GSRs and PIs after 6 hours of culturing were shown in Table 1 in form of mean ± standard deviation**.**

Based on the results of the test, the extract from the rhizome of *C. longa* displayed a considerable activity of growth inhibition on vegetative spores of *M. circinelloides*.

Table 1. The GSRs and the PIs represent the ability of spore germination and the speed of hyphae formation, respectively, of M. circinelloides upon exposure with the rhizome crude extract of C. longa after 6 hours of culturing (p < 0.05)

Negative control		**GSR (%)**	**PI**
		60.2±14.5	***15.2±1.3***
C. longa extract (mg/mL)	0.5	27.9 ± 9.5^{a}	1.6 ± 0.4^{a}
	1	22.7 ± 4.3^{b}	1.4 ± 0.2^{a}
	2	26.8 ± 14.5^{a}	1.3 ± 0.2^{a}
	3	26.3 ± 11.0^{a}	1.5 ± 0.3^{a}
	4	19.4 ± 7.1^{b}	1.6 ± 0.1^{a}
Amphotericin B (1 μg/mL)		4.0±1.6	<1.0

The rhizome crude extract of *C. longa* performed a significant inhibition on both GSRs and PIs, which refer to the capacity in the germination and the hyphae formation of the vegetative spores. Compare to the negative control group, the *C. longa* extract was able to reduce notably those quotients (by more than 50% with GSRs and 90% with PIs) right at the low concentrations. However, the effect likely to remain the same as the concentration of the extract increases.

The formation and development of the hyphae contribute greatly to the virulence of *M. circinelloides*, which is a non-dermatophyte, invasive fungus. The yeast-like type of growth has been reported to be less virulent than the type of hyphae growth [7, 8]. The link between dimorphism and virulence has been becoming a new and promising target to develop compounds against mucormycosis. *M. circinelloides* is one of the *Mucor* species displaying dimorphism, growing as multi-budded yeasts in anaerobic conditions, and as hyphae forming a mycelium in aerobic conditions. There are also chemical compounds inhibiting the mitochondrial function that can induce the yeast form, even in aerobic conditions, such as inhibitors of the electron transport chain, oxidative phosphorylation, and inhibitors of the synthesis of mitochondrial proteins [8, 30].

These observations suggest an active connection between aerobic respiration and the morphology of *Mucor* species.

The effects of the plant extraction on morphology and the growth type of *M. circinelloides* also were concerned due to their important distribution to the virulence of the fungus. In liquid YPG medium, the extract of *C. longa* seemed to induce the yeast-like growth of dimorphic *M. circinelloides* at the early stage of growth, despite being in aerobic culturing condition, especially in high concentration (Figure 1), resulting in very low PI values. This result suggests that the *C. longa* extract may contain one or some phytochemicals, which can affect the aerobic respiration of the fungal cell and might reduce the virulence of the *Mucor* fungus. This discovery also can make the extraction of *C. longa* be considered as a promising tool for control pathogenic fungal growth. However, further tests are needed to proceed for understanding better the impacts of the extract toward *M. circinelloides*, especially in the late growth stages.

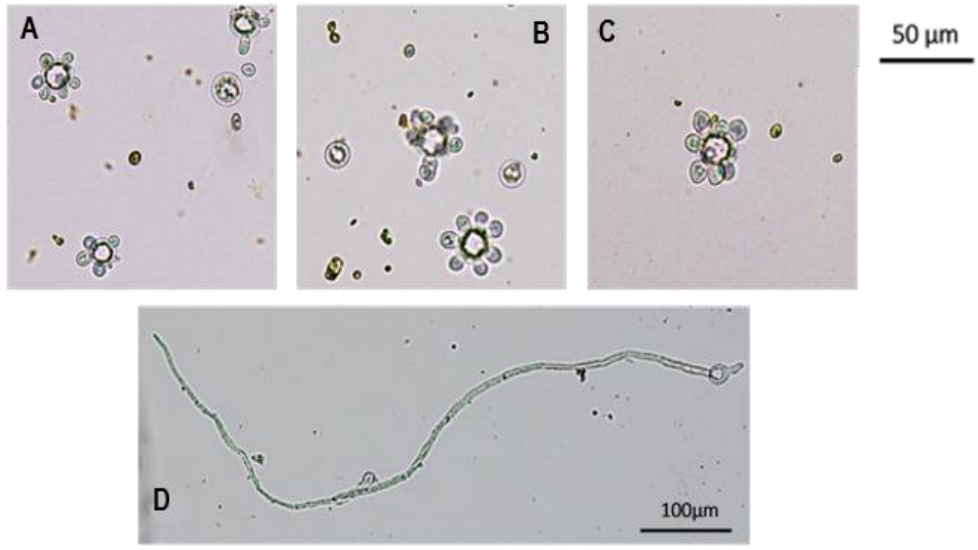

Figure 1. The multi-polar budding yeast of M. circinelloides after 7 hours exposure with the crude extract of C. longa (A-C), compared to the negative control (D)
***Concentration of the plant extracts:** A: 2.0 mg/mL, B: 3.0 mg/mL, C: 4.0 mg/mL*

2.3.2. Effects of the *C. longa* extract on the growth of *M. circinelloides* during the late stage

To investigate the effects of the plant crude extracts on the growth of *M. circinelloides* during the late growth stage (include arthrospores development, sporulation, and spreading [30], which take 3 to 5 days post-inoculation [31]) the evaluation by using agar well diffusion method was facilitated. Obtained results after 120 hours (5 days) of continuous culturing were shown in Table 2 in form of mean $\pm$ standard deviation (mm). Processed data were illustrated in Figure 2.

Table 2. Growth inhibitory activity of C. longa rhizome crude extract on M. circinelloides determined by agar well diffusion method (ZOI: zone of inhibition in mm) ($p < 0.05$)

Negative control		**ZOI (mm)**
		10.00
C. longa extract (mg/mL)	50	13.17±1.26
	100	14.67±1.53
	200	15.50±1.32
	300	16.17±1.04
	400	17.67±0.58[a]
Amphotericin B (400 μg/mL)		17.83±0.29[a]

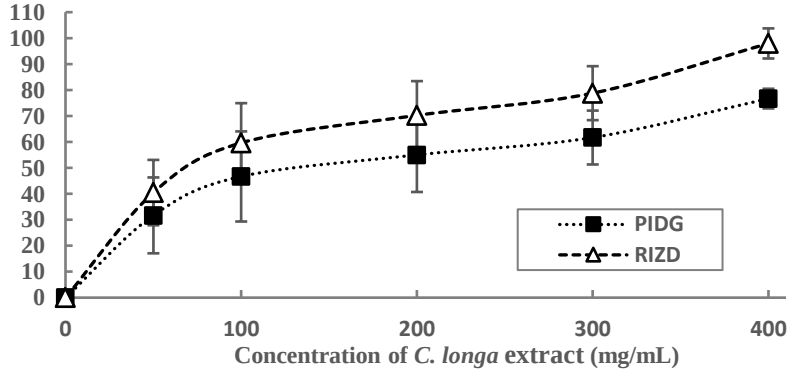

Figure 2. The PIDGs (%) and the RIZDs (%) of M. circinelloides upon exposure with the C. longa rhizome crude extract

The PIDGs evaluation represents the percentage of growth of inhibition of *M. circinelloides* upon exposure with the *C. longa* extract compared to the negative control; and the RIZDs evaluation represents the relative inhibitory ability of the extract on *M. circinelloides* compared to amphotericin B 400 μg/mL (100%). In a solid medium, *M. circinelloides* still exhibited a considerable susceptibility toward the crude extract of *C. longa*. At high concentrations (300 and 400 mg/mL), the extract even showed inhibitory activities that are similar to amphotericin B 400 μg/mL in the positive control. About the effect on the morphology, after a longer culturing period in a solid medium (YPG, agar plate, pH 4.5 for at least 120 hours), *M. circinelloides* still could develop and spread out in mycelium form under the exposure with the rhizome extract of *C. longa* (Figure 3).

These observations demonstrate that the extract *Curcuma* plant could induce the yeast-like growth of the *Mucor* fungus in the early stage but was not able to keep this mode in prolonged aerobic conditions. However, the rhizome *C. longa* extract generally showed promising antifungal activity against *M. circinelloides*. Moreover, due to the advantage of wide range antimicrobial characteristic and harmless to the low toxicity of using

C. longa externally, it is a great possibility to successfully develop new medical antiseptic products from the *Curcuma* plant extraction that can be very helpful in first aid as well as healing and preventing the wounds from infection. Furthermore, investigation for the combinations of different active compounds from different medicine plants might provide a huge potential source for innovative antifungal agent development.

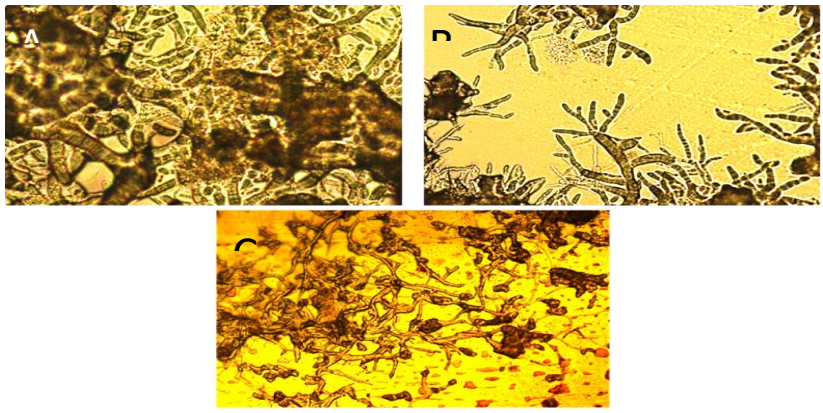

Figure 3. Morphology of M. circinelloides grown in the solid medium upon 120 hours exposure with C.longa rhizome crude extract (C), compared to the negative control (A) and AmBisome 400 µg/mL (B) (100X)

3. Conclusions

According to the results obtained in this study, it has been supported to conclude that: the rhizome crude extract from *C. longa* showed its ability to decrease the growth of the pathogenic microorganism in both early and late growth stages of *M. circinelloides*. The extract of *C. longa* likely to affect the hyphae - yeast transition of the fungi in the early stage. Those results suggest that the extract of *C. longa* could be used as a potential agent for future studies to find a good candidate for the treatment of mucormycosis.

Acknowledgements. This research is funded by the Ministry of Education and Training, Vietnam under grant number B2019-SPH-562-12.

REFERENCES

[1] C. E. Gonzalez, M. G. Rinaldi, and A. M. Sugar, 2002. Zygomycosis. *Infectious Disease Clinics of North America,* Vol. 16, Iss. 4. pp. 895-914.

[2] A. Katragkou, T. J. Walsh, and E. Roilides, 2014. Why is mucormycosis more difficult to cure than more common mycoses ? *Clinical Microbiology and Infection,* Vol. 20, Iss. 6, pp. 74-81.

[3] J. A. Ribes, C. L. Vanover-Sams, and D. J. Baker, 2000. Zygomycetes in human

disease. *Clinical Microbiology Reviews,* Vol. 13, Iss. 2. pp. 236-301.

[4] M. M. Roden *et al.*, 2005. Epidemiology and Outcome of Zygomycosis: A Review of 929 Reported Cases. *Clinical Infectious Diseases,* Vol. 41, Iss. 5, pp. 634-653.

[5] J. D. Jenks *et al.*, 2018. Rare mould infections caused by Mucorales, Lomentospora prolificans and Fusarium, in San Diego, CA: the role of antifungal combination therapy. *International Journal of Antimicrobial Agents,* Vol. 52, Iss. 5, pp. 706-712.

[6] L. Klingspor, B. Saaedi, P. Ljungman, and A. Szakos, 2015. Epidemiology and outcomes of patients with invasive mould infections: a retrospective observational study from a single centre (2005-2009). *Mycoses,* Vol. 58, Iss. 8, pp. 470-477.

[7] G. M. Gauthier, 2015. Dimorphism in Fungal Pathogens of Mammals, Plants, and Insects. *PLoS Pathogens,* Vol. 11, No. 2.

[8] S. C. Lee, A. Li, S. Calo, and J. Heitman, 2013. Calcineurin Plays Key Roles in the Dimorphic Transition and Virulence of the Human Pathogenic Zygomycete *Mucor circinelloides*. *PLoS Pathogens,* Vol. 9, Iss. 9.

[9] M. McIntyre, J. Breum, J. Arnau, and J. Nielsen, 2002. Growth physiology and dimorphism of *Mucor circinelloides* (syn. racemosus) during submerged batch cultivation. *Applied Microbiology Biotechnology,* Vol. 58, Iss. 4, pp. 495-502.

[10] C. H. Li *et al.*, 2011. Sporangiospore size dimorphism is linked to virulence of *Mucor circinelloides*. *PLoS Pathogens,* Vol. 7, Iss. 6.

[11] R. Laniado-Laborín and M. N. Cabrales-Vargas, 2009. Amphotericin B: side effects and toxicity. *Revista Iberoamericana de Micologia*, Vol. 26, Iss. 4, pp. 223-227.

[12] K. M. Nelson, J. L. Dahlin, J. Bisson, J. Graham, G. F. Pauli, and M. A. Walters, 2017. The Essential Medicinal Chemistry of Curcumin. *Journal of Medicinal Chemistry*, Vol. 60, Iss. 5, pp. 1620-1637.

[13] E. Ravindran, P. N., 2007. *The genus Curcuma. Boca Raton*. FL: Taylor & Francis.

[14] H. Chowdhury and T. B. Journal, 2008. In vitro screening of *Curcuma longa* L and its derivatives sa antifungal agents against Helminthosporrum oryzae and Fusarium solani. *Pesticide Research Journal*, Vol. 20, Iss. 1, pp. 6-9.

[15] M. key Kim, G. ja Choi, and H. S. Lee, 2003. Fungicidal property of *Curcuma longa* L. rhizome-derived curcumin against phytopathogenic fungi in a greenhouse. *Journal of Agricultural and Food Chemistry,* Vol. 51, Iss. 6, pp. 1578-1581.

[16] A. Apisariyakul, N. Vanittanakom, and D. Buddhasukh, 1995. Antifungal activity of turmeric oil extracted from *Curcuma longa* (Zingiberaceae). *Journal of Ethnopharmacology,* Vol. 49, Iss. 3, pp. 163-169.

[17] L. M. Damián-Badillo, R. Salgado-Garciglia, R. Elisa Martínez-Muñoz, and M. Manuel Martínez-Pacheco, 2008. Antifungal Properties of Some Mexican Medicinal Plants. *The Open Natural Products Journal,* Vol. 1, pp. 27-33.

[18] K. Neelofar, S. Shreaz, B. Rimple, S. Muralidhar, M. Nikhat, and L. A. Khan, 2011. Curcumin as a promising anticandidal of clinical interest. *Canadian*

Journal of Microbiology, Vol. 57, Iss. 3, pp. 204-210.

[19] C. Chen *et al.*, 2018. Antifungal activity, main active components and mechanism of *Curcuma longa* extract against Fusarium graminearum. *PLoS One*, Vol. 13, Iss. 3.

[20] R. Kumar Pundir and P. Jain, 2010. Comparative studies on the antimicrobial activity of black pepper (Piper nigrum) and turmeric (*Curcuma longa*) extracts. *International Journal of Applied Biology and Pharmaceutical Technology*, Vol. 1, Iss. 2, pp. 492-500.

[21] M. I. G. Roncero, 1984. Enrichment method for the isolation of auxotrophic mutants of Mucor using the polyene antibiotic N-glycosyl-polifungin. *Carlsberg Research Communications*, Vol. 49, Iss. 7, pp. 685-690.

[22] Q. Van Nguyen, T. H. Tran, T. N. Pham, D. Van Thuoc, V. D. Cao, and K. H. Boo, 2019. Inhibitory Effects of Bidens pilosa Plant Extracts on the Growth of the Bloom-Forming Alga Microcystis aeruginosa. *Water, Air, & Soil Pollution*, Vol. 230, Iss. 1.

[23] S. Vellanki *et al.*, 2018. *Mucor circinelloides*: Growth, Maintenance, and Genetic Manipulation. *Current Protocols in Microbiology*, Vol. 49, Iss. 1.

[24] CLSI, 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing Filamentous Fungi, Approved Standard, 2nd ed., CLSI document M38-A2. *950 West Valley Roadn Suite 2500,Wayne, Pennsylvania 19087, USA*.

[25] M. Schuster, S. Treitschke, S. Kilaru, J. Molloy, N. J. Harmer, and G. Steinberg, 2012. Myosin-5, kinesin-1 and myosin-17 cooperate in secretion of fungal chitin synthase. *The EMBO Journal*, Vol. 31, Iss. 1, pp. 214-227.

[26] J. M. Andrews, 2001. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, Vol. 48, Iss. suppl. 1, pp. 5-16.

[27] W. H. A. Wan Harun, A. Nordin, and F. Abdul razak, 2011. Determination of the percentage inhibition of diameter growth (PIDG) of Piper betle crude aqueous extract against oral Candida species. *Journal of Medicinal Plants Research*, Vol. 5, pp. 878-884.

[28] H. James Doughari, P. Alois Ndakidemi, I. Susan Human, and S. Benade, 2011. Antimicrobial susceptibility profile and effect of stem bark extracts of Curtisia dentata on multi-drug resistant verotoxic Escherichia coli and Acinetobacter spp. isolates obtained from water and wastewater samples. *Science and Technology Against Microbial Pathogens: Research, Development and Evaluation: Proceedings of the International Conference on Antimicrobial*, pp. 281-287.

[29] T. L. Lubbehusen, J. Nielsen, and M. McIntyre, 2003. Characterization of the *Mucor circinelloides* life cycle by on-line image analysis. *Journal of Applied Microbiology*, Vol. 95, Iss. 5, pp. 1152-1160.

[30] M. . de Hoog, G.S; Guarro, J.; Gene, J.; Figueras, 2000. *Atlas of clinical fungi (2nd ed.)*. Utrecht: Centraalbureau voor Schimmelcultures.

[31] C. V. Sciortino, 2017. *Atlas of Clinically Important Fungi*. Hoboken, NJ, USA: John Wiley & Sons, Inc., p. 106-100.