

IAI SPECIAL EDITION

RESEARCH ARTICLE

Antifungal properties of the extracts and fractions of black ear fungus (*Auricularia nigricans*)

Ika Kurnia Sukmawati^{1,2}, Anas Subarnas¹, Tina Rostinawati³, Sri Adi Sumiwi¹, Dede Saputra²

¹ Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Padjadjaran University, Sumedang, Indonesia

² Faculty of Pharmacy, Bhakti Kencana University, Bandung, Indonesia

³ Department of Pharmaceutical Biology, Faculty of Pharmacy, Padjadjaran University, Sumedang, Indonesia

Keywords

Antifungal
Auricularia nigricans
Disc diffusion
Microdilution

Correspondence

Tina Rostinawati
Department of Pharmaceutical Biology
Faculty of Pharmacy
Padjadjaran University
Sumedang
Indonesia
t.rostinawati@unpad.ac.id

Abstract

Background: Fungal infections are a common problem in tropical areas like Indonesia. Therefore, an alternative antifungal therapy is needed, focusing on black ear fungus (*Auricularia nigricans*), which contains flavonoids, alkaloids, phenolics/hydroquinones, monoterpenes, and sesquiterpenes with potential antifungal properties. **Objective:** This study aimed to evaluate the antifungal activity of various extracts and fractions derived from *Auricularia nigricans* against *Aspergillus flavus*, *Candida albicans*, and *Microsporium gypseum* by determining the Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC). **Methods:** Antifungal activities were assessed using disc diffusion and microdilution techniques. **Results:** The microdilution test against *Candida albicans* showed MIC values of 12,500 µg/mL, 6,250 µg/mL, 3,125 µg/mL, and 25,000 µg/mL for the fungus extract, n-hexane fraction, ethyl acetate, and methanol:water respectively, with MFC of each parameter exceeding 50,000 µg/mL. For *Microsporium gypseum*, the extract and n-hexane exhibited MIC of 6,250 µg/mL, while the methanol:water and ethyl acetate fractions had MIC of 12,500 µg/mL and 3,125 µg/mL with MFC >50,000 µg/mL and equal to 50,000 µg/mL, respectively. **Conclusion:** All the extracts and fractions demonstrated antifungal activity against *Candida albicans* and *Microsporium gypseum*, excluding the fungal sample of *Aspergillus flavus*.

Introduction

Infectious diseases are a significant health challenge in Indonesia, with fungal pathogens such as *Aspergillus flavus*, *Candida albicans*, and *Microsporium gypseum* being the predominant cause in tropical areas. National basic health research reports have indicated a concerning 20-25% increase in the prevalence of fungal infections (Feizia Huslina, 2017; Kemenkes, 2018).

Aspergillosis, a fungal infection caused by *Aspergillus flavus*, primarily affects the respiratory tract and can sometimes impact the skin and eyes, particularly in individuals with a compromised immune system. *Candida albicans* causes candidiasis, which can manifest for long or short periods of time, usually around the skin found between the toes, thigh folds, nails, armpits, and hair. *Microsporium gypseum* induces skin disease characterized by nail and hair destruction. Globally, these three fungal species respectively

account for approximately 1,000,000, 9,500,000, and 1,500,000 cases annually (Vandeputte *et al.*, 2012).

The treatment of fungal infections comprises both non-pharmacological and pharmacological approaches. Non-pharmacological strategies emphasize the maintenance of personal hygiene and immune system support. Pharmacological interventions are carried out by administering appropriate antifungal drugs such as ketoconazole, miconazole, itraconazole, flucytosine, terbinafine, and butenafine. These drugs have limitations, including poor penetration in certain tissues, a narrow spectrum of activity, certain severe side effects, and the emergence of fungal resistance. Consequently, there is an increasing demand for novel therapeutic approaches, and one such approach is exploring the potential of edible mushrooms such as black ear fungus (*Auricularia nigricans*) (Figure 1) (Kulkarni *et al.*, 2017).



Figure 1. Black ear fungus (*Auricularia nigricans*)

In Indonesia, there are 2,273 mushroom species, representing only about 0.15% of the total fungal diversity in the world. The antifungal activity of red ear mushrooms against *Aspergillus flavus*, *Candida albicans*, and *Microsporium gypseum* was reported in a study by Sukmawati et al. (Sukmawati et al., 2018). Phytochemical screening of the red ear fungus indicated the presence of alkaloids, tannins, and steroids/triterpenoids, suspected to possess antifungal properties. Similarly, black ear mushrooms have been reported to contain alkaloids, flavonoids, phenolic/hydroquinones, monoterpenes, and sesquiterpenes, with potential antifungal activity (Liana et al., 2015; Sukmawati et al., 2018). Therefore, this research aimed to evaluate the potential antifungal activity of black ear fungus (*Auricularia nigricans*) against the tested fungal pathogens.

Methods

Chemical and reagents

The main materials used in this research were *Auricularia nigricans*, potato dextrose agar (PDA) from MERCK, nystatin, 96% ethanol solvent, n-hexane, ethyl acetate, methanol, distilled water, DMSO solvent from MERCK, sodium hydroxide, gelatin, aliquots, Liebermann Bouchardat reagent, Dragendorff reagent, Mayer's reagent, magnesium powder (Mg), H₂SO₄, FeCl, NaCl, BaCl, and paper discs.

Afterwards, the collection of test plants, determination, extraction and fractionation of samples were conducted. *Auricularia nigricans* mushrooms were obtained from Cikole Market, Lembang Bandung, West Java. Next, the species determination was carried out at the Bandungense Herbarium (FMIPA) SITH ITB.

The mushroom collection goes through a series of processing stages, including wet sorting, washing, dry sorting, cutting into small pieces, drying, and grinding into powdered simplicia. The processed *Auricularia nigricans* samples were extracted via reflux using 96% ethanol. The thick extract is then fractionated to produce n-hexane, ethyl acetate, and water-methanol fractions.

Selection of test mushrooms

Candida albicans and the fungus *Microsporium gypseum* were obtained from the Microbiology Laboratory, Faculty of Pharmacy, ITB, while *Aspergillus flavus* was obtained from the UNPAD Microbiology Laboratory.

Fungal media used PDA, which was dissolved and sterilized by autoclaving at 121°C for 15 minutes.

Preparation of fungi suspension

One sample of test fungus was collected from the agar medium and mixed with 5 mL of sterile salt solution. The cell density was adjusted using a spectrophotometer produced by McFarland standard 0.5 at a wavelength of 530 nm to reach an absorbance of 0.08-0.1 (equivalent to 5x10⁶ cells per ml). The resultant sample was diluted by adding 0.1 mL total volume to 10 mL NaCl (1:100), followed by mixing 1 mL with 20 mL RPMI 1640/PDB medium (1:20) to obtain a final concentration of 5.0 x 10² to 2.5 x 10³ cells per mL.

Antifungal activity test

A 100 µL aliquot of gross domestic product (GDP) suspension was placed in the first column of the microplate as a negative control. Then, 5 µL of fungal suspension was added to 10 mL of PDB and vortex-stirred. A total of 100 µL of this mixture was put into the 2nd to 12th columns of the microplate. In the 12th column, 100 µL of antibiotic solution or extract of a certain concentration was added and homogenized. Starting from the 12th column, 100 µL was transferred to the 11th column, and this serial dilution continued until the 3rd column reached the lowest concentration. The microplates were incubated at 25°C for three periods of 24 hours each, and the absence of microbial growth was observed. The lowest concentration at which no visible microbial growth occurs is defined as the Minimum Inhibitory Concentration (MIC). A 5 µL aliquot from each clear well was transferred to a PDA and incubated at 25°C for three 24 h periods. The lowest concentration at which no microbial growth is observed is defined as the Minimum Fungicide Concentration (MFC). MIC is the lowest concentration that can still

provide obstacles to fungal growth, while MFC is the lowest concentration that can kill fungal growth.

Bioautography test

A total of 15 mL of PDA media was mixed with 0.2 mL of *Candida albicans* suspension and poured into a sterile Petri dish until it solidified. Pre-eluted thin layer chromatography (TLC) plates were placed on the surface of the agar medium, and after 30 min, they were removed. The agar area previously occupied by the TLC plate was incubated at 25°C for 24 hours to observe the inhibition zone (Syarifuddin, 2019).

Results

In this study, 21 kg of fresh black ear mushrooms were obtained from Cikole, Kec. Lembang, West Bandung Regency, West Java. Species determination was performed at the Herbarium Bandungense, SITH ITB, and the results confirmed the sample as black ear fungus (*Auricularia nigricans*). The fungus collections were thoroughly cleaned with water to remove impurities, drained, sliced into thin pieces, and dried using a combination of sunlight and air conditioning. Up to 3000 grams of dried black ear fungus simplicia were collected.

Phytochemical screening was conducted to determine the presence of specific compound groups in the *Auricularia nigricans* sample. This initial analysis provided valuable insights into the chemical

composition of the fungus simplicia. The results of the phytochemical screening showed that black ear mushrooms were positive for containing alkaloids, flavonoids and steroids. The maceration method was used to extract black ear fungus simplicia. After soaking for three days, the yield was 1.74%.

Fraction preparation was carried out using the liquid-liquid extraction (ECC) method with three solvents with different polarities, namely Methanol: water (2:8, polar), n-hexane (non-polar), and Ethyl acetate (semi-polar). The fractionation process produced the following results: a 30.2% hexane fraction, a 17.4% ethyl acetate fraction, and a 49.6% methanol:water fraction.

The disc diffusion antifungal activity test was designed to assess the activity of black ear fungus extracts and fractions against test fungi by measuring the resulting zone of inhibition (CLSI, 2008). The results of measuring the absorption of suspensions of *Aspergillus flavus* (0.087), *Candida albicans* (0.081), and *Microsporium gypseum* (0.088) were in accordance with the literature. The results of testing extracts and fractions of black ear fungus are presented in Table I.

From these results, it can be seen that the black ear fungus extract and fraction showed activity against *Candida albicans* and *Microsporium gypseum* at an average concentration of 100,000 µg/mL. This concentration was used as a stock solution in subsequent tests using the microdilution method to determine the MIC and MFC values.

Table I: Black ear fungus disc diffusion test results

Test mushroom	Concentration (µg/mL)	Average diameter of inhibition zone (mm) ± SD				
		Extract	F.N-hexane	F. Ethyl acetate	F. Methanol:water	Ketoconazole (10.000 µg/mL)
<i>Aspergillus flavus</i>	200,000	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	
	150,000	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	23.72±0.19
	100,000	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	
	50,000	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	
<i>Candida albicans</i>	200,000	15.77±0.35	9.73±0.31	9.00±0.79	6.00±0.00	
	150,000	11.47±0.82	8.47±0.25	7.50±0.36	6.00±0.00	17.37±0.45
	100,000	6.00±0.00	7.13±0.25	7.43±0.12	6.00±0.00	
	50,000	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	
<i>Microsporium gypseum</i>	200,000	10.03±0.32	8.33±0.21	8.17±0.31	7.23±0.12	
	150,000	8.73±0.15	6.30±0.20	7.63±0.29	6.70±0.10	11.73±0.35
	100,000	7.10±0.20	7.57±0.25	7.03±0.21	6.27±0.15	
	50,000	7.50±0.20	7.03±0.21	6.93±0.15	6.00±0.00	

*Average of 3 repetitions; Disc diameter 6 mm

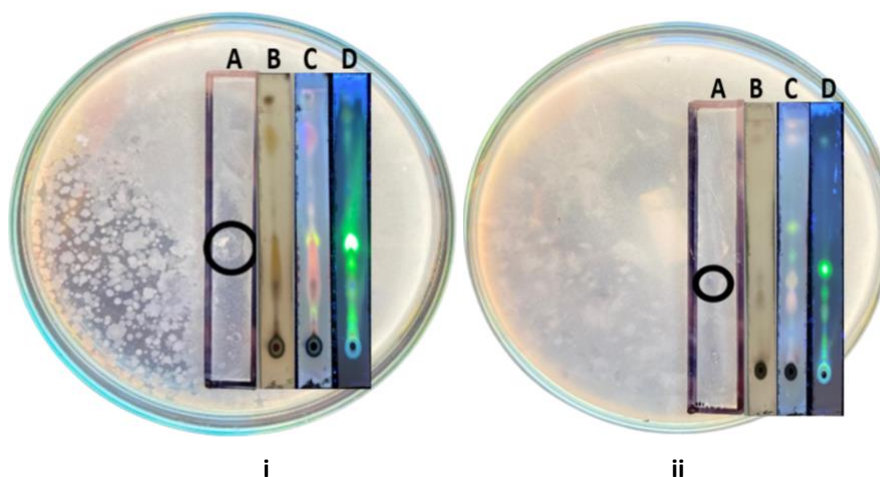
The antifungal activity test used the microdilution method, which aims to determine the MIC and MFC of black ear fungus extracts and fractions against *Aspergillus flavus*, *Candida albicans* and *Microsporium gyseum*. The results of antifungal activity tests carried out on extracts and fractions of black ear fungus against *Aspergillus flavus*, *Candida albicans*, and *Microsporium gyseum* can be seen in Table II. Bioautography was

carried out to classify secondary metabolite compounds that have antifungal activity. The results of the bioautographic test of the extract and ethyl acetate fraction against *Microsporium gyseum* are presented in Figure 2. Based on the monitoring results, a clear zone with Rf values of 0.38 and 0.40 was observed in the media for the extract and ethyl acetate fraction.

Table II: Results of the black ear mushroom microdilution test

Test mushrooms	Test substance	MIC ($\mu\text{g/mL}$)	MFC ($\mu\text{g/mL}$)
<i>Aspergillus flavus</i>	Extract	>50,000	>50,000
	F. N-hexane	50,000	>50,000
	F. Ethyl acetate	5,000	>50,000
	F. Methanol:water	>50,000	>50,000
	Ketoconazole	39,063	5,000
<i>Candida albicans</i>	Extract	12,500	>50,000
	F. N-hexane	6,250	>50,000
	F. Ethyl acetate	3,125	>50,000
	F. Methanol:water	25,000	>50,000
	Ketoconazole	39,063	312,5
<i>Microsporium gyseum</i>	Extract	6,250	>50,000
	F. N-hexane	6,250	>50,000
	F. Ethyl acetate	3,125	50,000
	F. Methanol:water	12,500	>50,000
	Ketoconazole	39,063	2,500

MIC = Minimum Inhibitory Concentration; MFC = Minimum Fungicide Concentration



i. Results of extract bioautography test against *Microsporium gyseum*

ii. Results of the Ethyl-Acetate Fraction Bioautography Test Against *Microsporium gyseum*

Information:

- Results of KLT monitoring of *Microsporium gyseum*
- The results of TLC monitoring of H₂SO₄ spots visually
- TLC monitoring results for H₂SO₄ spotting at UV 366 nm
- TLC monitoring results for the appearance of Liebermann-Burchard spots at UV 366 nm

Figure 2: Bioautography test results

Discussion

The ingredients used are black ear mushrooms sourced from Cikole, Kec. Lembang, West Bandung Regency, West Java. The results of the determination carried out at the Bandungense Herbarium, SITH ITB, stated that the sample was a black ear fungus (*Auricularia nigricans*). Black ear mushrooms are cleaned with water to remove dirt, drained, and then reduced in size. Reducing the size aims to speed up the drying process. Drying is carried out using direct sunlight and air conditioning. The dried simplicia is then ground into powder by blending and then weighing the dry powder. Simplicia purification is carried out to reduce the size of the simplicia so that the surface area in contact with the filter during the extraction process is wider and the extraction process for the chemical content contained in the simplicia is more optimal. Because treating the species affects the bioactive molecules that are extracted during the extraction process, it is crucial to do so before extracting secondary metabolites. By oxidative, enzymatic, or polymerization processes, the chemicals in the sample can be rapidly broken down (Yenn et al., 2018).

Extraction of black ear fungus simplicia was carried out using the maceration method. The maceration method of cold extraction was selected due to its ease of use and the long period of contact between the simplicia and the solvent, which, in this case, is 96% ethanol. Fraction preparation was carried out using the liquid-liquid extraction (ECC) method. ECC was chosen because the method is very simple, fast and easy. Additionally, three different solvents were selected to attract polar, non-polar, and semi-polar compounds in black ear fungus. The chemicals extracted from the extract are selectively affected by the organic solvent utilized. To distinguish between molecules that were soluble in water and those that were soluble in fat, this study employed organic solvents with different levels of polarity, including n-hexane, methanol:water, and ethyl acetate (Khodijah et al., 2023)

Black ear fungus contains more polar compounds, as can be seen from the yield, which is more absorbed in methanol:water. In addition, many adsorbed non-polar compounds can be observed, as indicated by the number of results with n-Hexane, and the least amount of semi-polar compounds, as observed in the results with Ethyl-acetate. The yield value shows how much content can be extracted by the solvent, expressed as a percentage. According to Syari & Aprilla's research in 2022, the diameter of the inhibition zone can be categorized as weak if the obstacle is <5 mm, medium if the obstacle is 5-10 mm, strong if the obstacle is 10-20 mm and very strong if the obstacle is > 20 mm (Syari & Aprilla, 2022). The concentrations of extracts and fractions used in the

disc diffusion test were 50,000 µg/mL, 100,000 µg/mL, 150,000 µg/mL, and 200,000 µg/mL.

From Table I, it is known that extracts and fractions of black ear fungus with concentrations of 50,000 µg/mL, 100,000 µg/mL, 150,000 µg/mL and 200,000 µg/mL did not show activity against *Aspergillus flavus*, judging from the absence of an inhibition zone formed. In contrast, ketoconazole, used as a comparison at a concentration of 10,000 µg/mL, showed significant activity, with an average inhibition zone measuring 23.72 mm.

Black ear fungus extracts and fractions have strong inhibitory activity against the growth of *Candida albicans*, as seen from the inhibition zone formed. Extracts with a concentration of 150,000 µg/mL and 200,000 µg/mL had an average inhibition zone diameter of 11-15 mm. The n-hexane and ethyl acetate fractions with concentrations of 100,000 µg/mL, 150,000 µg/mL, and 200,000 µg/mL had moderate inhibitory activity against the growth of *Candida albicans* with an average inhibitory zone diameter of 7-9 mm. On the other hand, an average inhibition zone of 17.37 mm indicates the activity of the comparison ketoconazole at a concentration of 10,000 µg/mL.

Black ear mushroom extracts and fractions have activity against *Microsporium gyseum*, as seen from the inhibition zone formed. Extracts at concentrations of 50,000 µg/mL, 100,000 µg/mL, 150,000 µg/mL, and 200,000 µg/mL have moderate inhibition of fungal growth with an average inhibitory zone diameter of 7-10 mm. The n-hexane, water-methanol and ethyl acetate fractions at concentrations of 50,000 µg/mL, 100,000 µg/mL, 150,000 µg/mL, 200,000 µg/mL had a moderate inhibitory activity with an average inhibition zone diameter of 6-8 mm. Conversely, at 10,000 µg/mL, the comparison ketoconazole demonstrated strong inhibition of growth, with an average diameter of the inhibition zone of 11.73 mm. The antifungal activity is thought to be caused by the secondary metabolites contained in black ear fungus, namely flavonoids (Moulishankar & Lakshmanan, 2020).

Flavonoids, for example, are secondary metabolites with antifungal properties found in extracts and fractions of black ear mushrooms that exhibit inhibitory activity against fungal growth. Flavonoids can have antifungal activity up to six times higher than what is now considered normal in certain situations. The results of the microdilution test indicated the MIC and MFC of black ear mushroom (*Auricularia nigricans*) against *Candida albicans* as follows: MIC for the extract is 12,500 µg/mL, for the n-hexane fraction 6,250 µg/mL, for the ethyl acetate fraction 3,125 µg/mL, and for the methanol fraction in water 25,000 µg/mL, all with MFC values > 50,000 µg/mL.

According to Aligiannis et al., antifungal MIC classification for plant-derived extracts categorizes MIC values as follows: <500 µg/mL indicates strong antifungal activity, 500-1500 µg/mL suggests moderate extract antifungal activity, and >1500 µg/mL signifies weak antifungal activity. Therefore, the black ear mushroom demonstrates weak inhibitory activity against the growth of the tested fungus.

The bioautographic test was conducted to identify the class of secondary metabolite compounds with antifungal activity. The results suggest that the active compounds belong to the steroid group, identifiable by a colour change to green spots in both the extract and ethyl acetate fraction, with Rf values of 0.38 and 0.40. Steroid compounds can inhibit fungal growth by affecting the cytoplasm and disrupting the growth and development of fungal spores. Furthermore, steroids can function as antifungals because the lipophilic properties of steroids can inhibit spore germination in fungi (Rieska Alfiah et al., 2015).

Conclusion

In conclusion, the results showed that the extract and fraction of black ear fungus (*Auricularia nigricans*) had antifungal activity against *Candida albicans* and *Microsporum gypseum* but had no activity against *Aspergillus flavus*. The results of the bioautographic test showed that the compound that had growth-inhibiting activity against the test fungus *Microsporum gypseum* was a steroid.

Acknowledgement

The author would like to express gratitude to the Directorate General of Higher Education for the grants provided, and to the Faculty of Pharmacy at Padjadjaran University and LPPM Bhakti Kencana University for their assistance.

Source of funding

Directorate General of Higher Education

References

- Khodijah, R., Haryani, Y., Teruna, H. Y., & Hendra, R. (2023). Antibacterial properties of *Pyrrrosia longifolia* extracts. *Pharmacy Education*, **23**(2), 168–173. <https://doi.org/10.46542/pe.2023.232.168173>
- Kulkarni, C. P., Pandey, A., & Patil, R. C. (2017). Antifungal and phytochemical properties of *Tinospora cordifolia*, *Azadirachta indica* and *Ocimum sanctum* leaves extract. *Journal of Medicinal Plants Studies*, **5**(5), 23–26.
- Liana, M., Fitrianiingsih, S. P., Mulqie, L., & Farmasi, P. (2015). Characterization of simplicia and ethanol extract of black ear fungus (*Auricularia polytricha*). *Prosiding Farmasi*, **1**(2).
- Moulishankar, A., & Lakshmanan, K. (2020). Data on molecular docking of naturally occurring flavonoids with biologically important targets. *Data in Brief*, **29**, 105243. <https://doi.org/10.1016/j.dib.2020.105243>
- Rieska Alfiah, R., Khotimah, S., Turnip, M., Studi Biologi, P., Mipa, F., Tanjungpura, U., & Hadari Nawawi, J. H. (2015). Effectiveness of methanol extract of Sembung Rambat (*Mikania micrantha* Kunth) leaves against the growth of the fungus *Candida albicans*. *Jurnal Protobiont*, **4**(1), 52–57. <https://jurnal.untan.ac.id/index.php/jprb/article/viewFile/8735/8710>
- Sukmawati, I. K., Yuniarto, A., & Rakhmawati, D. (2018). Antifungal activity of extract and fraction of *Auricularia Auricular* on *Candida albicans*, *Microsporum gypseum*, and *Aspergillus flavus*. *Asian Journal of Pharmaceutical and Clinical Research*, **11**(Special Issue 1), 141–145. https://doi.org/10.22159/ajpcr.2018.v11s1.2659_1
- Syari, D. M., & Aprilla, C. (2022). Antibacterial activity of Tehtehan plant leaf extract (*Acalypha siamensis*) against *Staphylococcus aureus* bacteria using the disc method. *Cakram. Journal Ilmiah Farmasi Imelda*, **5**(2). <https://doi.org/10.52943/jifarmasi.v5i2.776>
- Syarifuddin, A., Sulistyani, N., & Kintoko -. (2019). Profil KLT-bioautografi dan densitometri fraksi teraktif (Isolat Kp13) dari bakteri rizosfer kayu putih. *Jurnal Farmasi Sains Dan Praktis*, **5**(1), 27–33. <https://journal.unimma.ac.id/index.php/pharmacy/article/view/2291/1436>
- Vandeputte, P., Ferrari, S., & Coste, A. T. (2012). Antifungal resistance and new strategies to control fungal infections. *International Journal of Microbiology*, **2012**, 713687. <https://doi.org/10.1155/2012/713687>
- Yenn, T. W., Ring, L. C., Zahan, K. A., Rahman, M. S. A., Tan, W. N., & Alaudin, B. J. S. (2018). Chemical composition and antimicrobial efficacy of *Helminthostachys zeylanica* against foodborne *Bacillus cereus*. *Natural Product Sciences*, **24**(1), 66–70. <https://doi.org/10.20307/nps.2018.24.1.66>