BIODIVERSITAS
Volume 24, Number 5, May 2023
Pages: 2547-2555

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240506

Anticandidal and antioxidant potencies of endophytic fungi associated with *Tinospora crispa*

AHMAD FATHONI[1,2,♥♥], SUMI HUDIYONO[1,♥], ANTONIUS HERRY CAHYANA[1], MUHAMMAD ILYAS[3], ISMU PURNANINGSIH[4], ANDRIA AGUSTA[2,5]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Jl. Lingkar Kampus Raya, Pondok Cina, Beji, Depok 16424, West Java, Indonesia. Tel.: +62-217-270027, ♥email: sumi.hudiyono@sci.ui.ac.id

[2]Research Center for Pharmaceutical Ingredient and Traditional Medicine, National Research and Innovation Agency. Jl. Raya Jakarta-Bogor Km 46, Cibinong, Bogor 16911, West Java, Indonesia. ♥♥email: ahma060@brin.go.id

[3]Research Center for Biosystematics and Evolution, National Research and Innovation Agency. Jl. Raya Jakarta-Bogor Km. 46 Cibinong, Bogor 16911, West Java, Indonesia

[4]Directorat of Scientific Collection Management, National Research and Innovation Agency. Gedung BJ Habibie, Jl. M.H. Thamrin No. 8, Central Jakarta 10340, Jakarta, Indonesia

[5]Faculty Medicine, Universitas Malahayati. Jl. Pramuka No. 27, Kemiling Permai, Bandar Lampung 35152, Lampung, Indonesia

Manuscript received: 5 March 2023. Revision accepted: 8 May 2023.

Abstract. *Fathoni A, Hudiyono S, Cahyana AH, Ilyas M, Purnaningsih I, Agusta A. 2023. Anticandidal and antioxidant potencies of endophytic fungi associated with* Tinospora crispa*. Biodiversitas 24: 2547-2555.* Natural products including endophytic fungi, have opportunities for medicinal uses as anticandidal and antioxidant agents. This study aimed to investigate the anticandidal and antioxidant activities of endophytic fungi isolated from Brotowali (*Tinospora crispa* (L.) Miers ex Hook.fil. & Thomson) using disc diffusion, thin layer chromatography (TLC) dot-blot, and microdilution methods. Eighty extracts were screened for anticandidal activity against *Candida albicans* (C.P.Robin) Berkhout and antioxidants against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Disc diffusion results showed that 38 endophytic fungi had an anticandidal activity with an inhibition zone diameter (IZD) of 7.00±00 to 18.1± 0.29 mm and 4 extracts with minimum inhibitory concentration (MIC) values of 32-64 µg/mL (strong, MIC<100). The TLC dot-blot results showed that 23 extracts had antioxidant activity and 8 extracts were very strong with an inhibitory concentration of 50% (IC_{50}) values of 7.76±0.77 to 25.54±1.37 µg/mL, while the antioxidant activity index (AAI) values ranged from 2.01±0.10 to 6.64±0.63 (very strong, AAI>2). Results of the Pearson correlations coefficient between the total phenolic content (TPC) value and AAI value, revealed that there was a low correlation, while the correlation of IZD and MIC as anticandidal has a moderate negative (the r value 0.332, and -0.673, respectively (P<0.01). Purification of bioactive compounds is necessary, and endophytic fungi associated with *T. crispa* can serve as a potential source of natural antioxidants and anticandidal agents.

Keywords: Anticandidal, antioxidant, *brotowali*, endophytic fungi, *Tinospora crispa*

INTRODUCTION

Endophytes are commonly found in most plant species. They live within plant tissues both throughout their life and during certain periods of their life cycle without causing visible damage or morphological changes to their host plants, thus providing benefits for the host plant while also benefiting from this interaction (Alam et al. 2021). These microorganisms of endophytic, include bacteria and fungi (Kandel et al. 2017; Alam et al. 2021) and usually coexist with pathogens. Endogenous microbiota can be divided into obligate and facultative categories according to their colonization behavior. Obligate strains inhabit plants throughout their life cycle. They typically use or modify metabolites and plant products for survival and propagate across plant generations by vertical transmission (Gouda et al. 2016). Whereas facultative endophytes colonize plants at certain stages of the life cycle but live outside the plant at other stages to form direct rhizosphere soil associations of the host plant (Lorena et al. 2021).

One of the medicinal plants as a host of endophytic is Brotowali (*Tinospora crispa* (L.) Miers ex Hook.fil. & Thomson. This plant is found in many rainforests in Asia and Africa. The plant has been used traditionally in the treatment of various diseases and to maintain health. Its pharmacological activities include anti-inflammatory, antioxidant, immunomodulatory, cytotoxic, antimalarial, cardioprotective, and anti-diabetic activities (Ahmad et al. 2016). In previous studies, endophytic fungi could be used as antibacterial against *Staphylococcus aureus* Rosenbach 1884 and *Escherichia coli* E with moderate to strong activity (Fathoni et al. 2021).

On the other hand, natural products derived from endophytic fungi have various benefits in medicinal use, including anticandidal and antioxidants agents (Agusta et al. 2013; Praptiwi 2018; Wulansari et al. 2016; Fathoni et al. 2022a). In addition, endophytic fungi are also widely known as a storehouse of bioactive compounds useful in various fields including medicinal, pharmaceutical, agricultural, and industrial purposes (Gouda et al. 2016; Chutulo and Chalannavar 2018; Rana et al. 2020; Wen et al. 2022; Fathoni et al. 2022a).

Currently, *Candida albicans* (C.P.Robin) Berkhout is the most common fungal pathogen in humans. Due to the

development of drug resistance, there is an urgent need for new antifungal agents to effectively fight *C. albicans* (Zida et al. 2017). In a previous study, a secondary metabolite from fungal endophyte *Diaporthe* sp. GNBP-10 associated with *Uncaria gambir* (W.Hunter) Roxb that produced (+)-2,2'-epicytoskyrin have moderate antifungal activity against 22 yeast strains and three filamentous fungi including against *C. albicans* (Wulansari et al. 2016).

Besides, it is important to find effective anticandidal, currently, research is also developing to obtain natural antioxidants to treat degenerative diseases. Endogenous and exogenous sources cause oxidative stress processes originating from free radicals of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Environmental pollution, radiation, polluted water, pesticides, heavy metals, stress, and oxygen metabolism in living cells can cause the formation of free radicals in the human body. Free radicals are highly reactive forms of oxygen that destroy the cells of organisms. This can lead to cardiovascular disease, cancer, cataracts, diabetes, and many other diseases (Atasoy and Yücel 2021). In this case, antioxidants from dietary sources are needed to prevent excessive free radical damage (Li et al. 2014).

Endophytic fungi promote host plant growth by directly producing secondary metabolites, which increase the resistance of plants to biotic and abiotic stress including oxidative stress (Wen et al. 2022). In a previous study, nigerloxin, a fungal metabolite produced by *Aspergillus niger* Tiegh has effective antioxidant in-vitro assays including against free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Suresha and Srinivasan 2013). Another fungal metabolite, pestacin from *Pestalotiopsis microspora* (Speg.) G.C.Zhao & Nan Li, has moderate antifungal and strong antioxidants (Harper et al. 2003). Previous studies reported the bioactivity of endophytic fungus isolated from *T. crispa* that could be used as a source of antibacterial (Fathoni et al. 2021, 2022c). Still, they have never been investigated for anticandidal and antioxidant potencies. Therefore, this research aimed to investigate the anticandidal and antioxidant activities of endophytic fungi isolated from *T. crispa*.

MATERIALS AND METHODS

Endophytic fungal extracts

Eighty endophytic fungi were isolated from the stems, leaves, and petioles of *T. crispa*. These fungi were identified based on morphological characteristics (Ilyas et al. 2019). The isolated fungi were cultured on PDB for 21 days under static conditions in a dark room at a temperature of 25°C. Fungal cultures were extracted using ethyl acetate (EtOAc), and the resulting extracts were concentrated using rotatory evaporator (Fathoni et al. 2021).

Identification of selected endophytic fungi

The representative potential strains were then selected for further identification using molecular approach. Molecular identification was conducted by analyzing the DNA sequence of an internal transcribed spacer (ITS) region (Fathoni et al. 2022b). The total fungal genomic DNA was isolated using Nucleon PhytoPure, plant, and fungal DNA extraction kits (GE Healthcare) according to the manufacturer`s instructions. DNA amplification of the ITS rDNA region was performed by polymerase chain reaction (PCR). PCR amplification was performed in 25-µL reaction mixtures containing 10 µL distilled water, 12,5 µL GoTaq Green Master Mix (Promega), 0.5 µL DMSO, 0,5 µL each primer (10 pmol), and 1 µL (5 to 10 ng) extracted genomic DNA as a template. The primer set of ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 c(5`-GGAAGTAAAAGTCGTAACAAGG-3`) was used to amplify approximately 550 nucleotides from ITS1 and ITS 2 including 5.8S rDNA (White et al. 1990). Amplification was performed using a TaKaRa PCR and followed the program of amplification according to Napitupulu et al. (2019). The PCR products were then subjected to sequence analysis. Initial phylogenetic tree construction of selected strains was conducted by editing the raw sequence data usingChromasPro(http://www.technelysium.com.au/ChromasPro.html). The assembled sequences were aligned with those downloaded from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nlh.gov/) using the Muscle (http://www.ebi.ac.uk/Tools/msa/muscle). The phylogenetic analysis of sequence data were done based on the neighbor-joining (NJ) method (Saitou and Nei 1987) using Molecular Evolutionary Genetics Analysis (MEGA) version 7 program (Kumar et al. 2016). The reliability of each branch was evaluated by bootstrapping with 1000 resampling.

Anticandidal activity using disc diffusion assay

For an anticandidal test, a filter paper disc containing the extracts was placed on an agar surface (Balouiri et al. 2016). The 6 mm sterile disc paper was soaked in 10 µL of extract (10 mg/mL in acetone), then dried in laminar airflow. A total of 10 µL acetone and methanol were used as negative controls and 10 µL nystatin (1 mg/mL in methanol) as a positive control. Then evenly spread 100 µL of *C. albicans* grown previously (in SDB medium, room temperature, cultivated in 48 hours) on PDA agar in a Petri dish. The paper disc containing the sample was then transferred to the surface of a PDA medium containing *C. albicans* and incubated at room temperature for 48 hours, in dark conditions. The activity of extract as anticandidal with the formation of clear zones or the diameter of inhibition of *C. albicans* growth was measured in mm.

Anticandidal activity for determining MIC value

The minimum inhibitory concentration (MIC) value for anticandidal activity, used the broth dilution method with serial concentration Balouiri et al. (2016). Active extracts were dissolved in 2.5% dimethyl sulfoxide (DMSO) and tested against *C. albicans*. One hundred microliters of the extract were transferred into the first tube and then serially diluted to make concentrations of 32, 64, 128, and 256 µg/mL in triplicate. These samples were then placed in a 96-well microplate and 100 µL of candidal suspension, containing approximately 10^6 CFU/mL, was added to each well. The microplate was incubated at 25°C for 48 hours

and the anticandidal test was conducted in triplicate. After incubation, ten microliters of 4 mg/mL sterile MTT was added to each well resulting in a purple color if candidal growth occurred. The lowest concentration that prevented visible growth of the microorganism, or the absence of color change, was considered as the MIC value of the sample that inhibited microorganism growth.

Antioxidant activity using TLC Dot-Blot assay

The extracts were dissolved in acetone to make a final concentration of 10 mg/mL. Ten microliters of extracts were transferred on a TLC plate and developed using dichloromethane-methanol (10:1). Separated chemical compounds were monitored under UV light of 254 and 366 nm and phenolic chemical were detected by spraying with Folin reagent (Fathoni et al. 2021).

Ten microliters of 10 mg/ml extracts were transferred to TLC plates and evaluated for antioxidants by using TLC-DB dot blot assay according to Fathoni et al. (2022a). After drying, the TLC plate was sprayed with 0.2 mg/mL DPPH in methanol and then incubated at room temperature for 5-10 minutes in dark conditions. A white-yellow zone around the extracts against a purple background on the TLC plate indicated the antioxidant activity of the samples.

Antioxidant activity for determining IC_{50} values of DPPH

Antioxidant activity for determining IC_{50} values of DPPH was carried out according to Fathoni et al. (2022b). The extracts were dissolved in Methanol with 1.25% dimethyl sulfoxide (DMSO) and were tested for antioxidant activity as DPPH free radical scavenging. One hundred microliters of the extract were transferred into the first tube and then serially diluted to make the concentrations of 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, and 128 µg/mL in triplicate at 96-well microplate were added to 100 µL of 61.5 µl/mL DPPH in methanol then incubated for 90 minutes at room temperature, in dark condition. The antioxidant activity of the extract was conducted in triplicate. The blank was methanol, while the positive control was catechin. The absorbance was observed at 517 nm using Varioskan Flash (Thermo Scientific). Percent inhibition concentration (IC) was calculated as follows:

$$IC\ (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) x100\%$$

Where: $A_{control}$ = the absorbance of DPPH without samples; A_{sample} = the absorbance of DPPH along with different concentrations of extracts. The IC_{50} values of DPPH were obtained from linear calibration between the percentage of inhibition and sample concentration. The antioxidant activity index (AAI) was obtained by the following formula:

$$AAI = \frac{\text{The final concentration of DPPH}}{IC_{50}}$$

Correlation test between TPC, anticandidal and antioxidant activities

The correlation test of TPC, anticandidal and antioxidant activities was performed by Pearson's correlation ($P<0.01$).

Statistical analysis

The statistical analysis of variance of both IC_{50} values toward DPPH radical free scavenging, IDZ against *C. albicans* was performed by multiple ranges of Duncan tests using SPSS 16.0. The experiment was performed in triplicate. Data were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Morphological identification of selected fungi

The results of morphological analysis showed that selected fungi number 18, 45, 57, 38, 43, and 52 were identified as *Phomopsis* sp. (TcTd1Bd-1), *Phomopsis* sp. (TcBt1Bo-4), *Epicoccum* sp. (TcDn1Bo-2), *Nigrospora* sp. (TcBt1Be-2), *Phomopsis* sp. (TcBt1Bo-2), and *Neofusicoccum* sp. (TcBt2Bo-1), respectively (Figure 1).

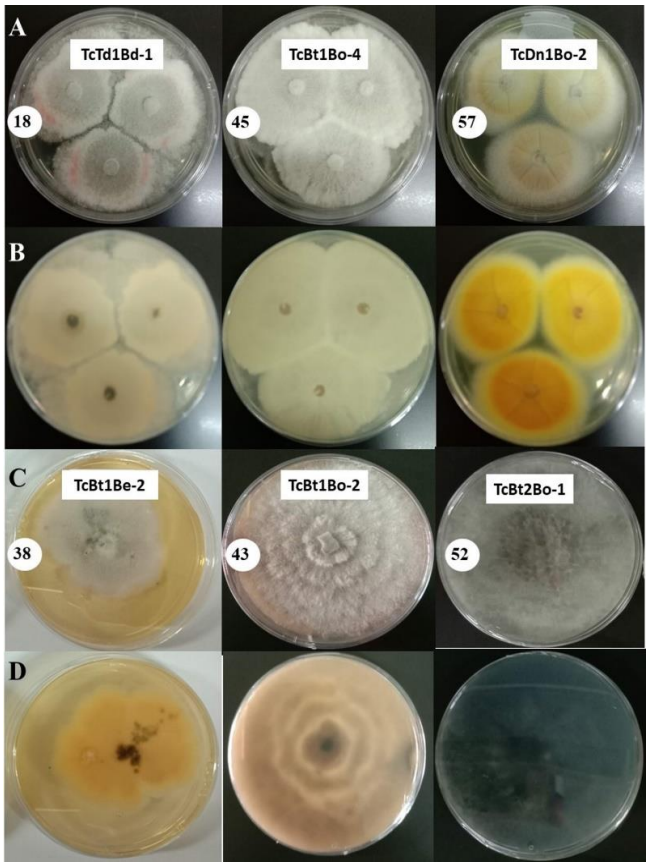

Figure 1. Pure cultures of selected endophytic fungi on PDA: A. No. 18 (*Phomopsis* sp.), 45 (*Phomopsis* sp.), 57 (*Epicoccum* sp.) at front view; B. No. 18, 45; 57 at back view; C. No. 38 (*Nigrospora* sp.), 43 (*Phomopsis* sp.), 52 (*Neofusicoccum* sp.) at front view; D. No.38, 43 and 52 at back view

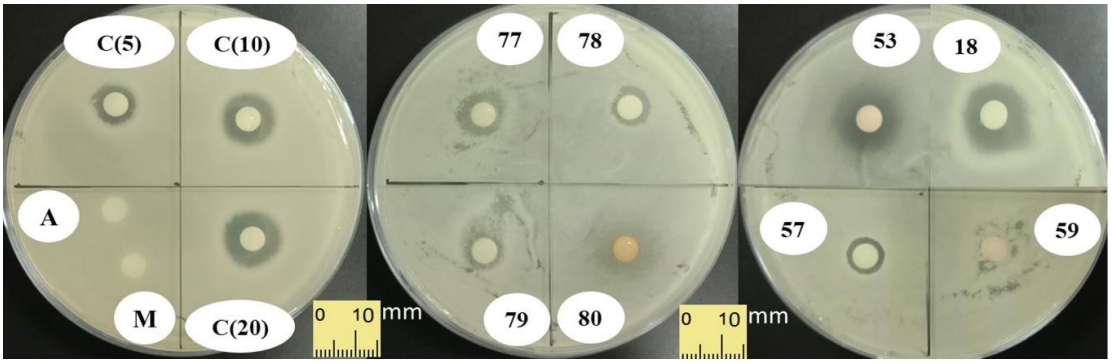

Figure 2. Disc diffusion assay of some endophytic fungi extracts against *C. albicans*. Note: C(5): 5 µL of 1 mg/mL nystatin; C(10): 10 µL of 1 mg/mL nystatin; C(20): 20 µL of 1 mg/mL nystatin; A: 10 µL acetone; M: 10 µL MeOH; 77: sample extract No.77; 78: sample extract No.78; 79: sample extract No.79; 80: sample extract No.80; 53: sample extract No.53; 18: sample extract No.18; 57: sample extract No.57; 59: sample extract No.59

Anticandidal activity of extracts of endophytic fungi

A total of 37 endophytic fungi out of 80 extracts were found active as anticandidal using a disc diffusion assay (Figure 2). Table 1 shows that some extracts had a weak anticandidal activity based on MIC value: >256 µg/mL (26 extracts), moderate: 100 µg/mL <MIC<256 µg/mL (7 extracts), and strong: MIC<100 µg/mL (4 extracts) with inhibition zone values varying from 7.00 ±0.00 to 18.17 ± 0.29 mm. The strong extracts with a MIC value of 32.00 µg/mL were *Phomopsis* sp. TcTd1Bd-1 (No. 18), *Epicoccum* sp. TcDn1Bo-2 (No. 57), and *Phomopsis* sp. TcBt1Bo-4 (No. 45).

Antioxidant activity of extracts of endophytic fungi

A total of 23 of the 80 endophytic fungi extracts were found to be active as antioxidants using the TLC Dot-Blot assay (Figure 3). In addition, the active extracts were further tested by TLC bioautography as well as a microdilution test for determining IC_{50} and AAI values. The active antioxidant compounds have shown a yellow spot with a purple background after spraying with the DPPH reagent as well as shown as phenolic compounds (dark color) after spraying with the Folin reagent (Figures 4 and 5).

Table 2 shows that several extract samples had weak antioxidants based on AAI values: AAI< 0.5 (2 extracts), moderate: 0.5<AAI < 1.0 (3 extracts), strong: 1.0<AAI<2.0 (10 extracts), and very strong: > 2.0 (8 extracts). The active extracts had IC_{50} values ranging from 7.76±0.77 to 393.51±21.28 µg/m. Only three extracts had a very strong antioxidant activity with AAI values of 6.04±0.61 to 6.64±0.63 µg/mL, namely *Phomopsis* sp. TcBt1Bo-2 (No.43), *Nigrospora guilinensis* Mei Wang & L.Cai TcBt1Be-2 (No.38), and *Neofusicoccum grevilleae* Crous & R.G.Shivas TcBt2Bo-1 (No.52).

Correlation test between TPC, anticandidal and antioxidant activities

Table 3 shows Pearson correlation value that the TPC variable had a weak positive correlation in proportion to the increase in the AAI value (r-value 0.332, P <0.01) and a weak negative correlation or inversely proportional to the MIC value in the very weak category (r value -0.029). Meanwhile, the value of the inhibition zone diameter of anticandidal had a moderate negative correlation with the MIC value of *C. albicans* (r-value -0.673, P<0.01). The higher the inhibition zone diameter, the more moderate the negative correlation of the MIC value, or the better the anticandidal activity of the extract.

Table 1. IDZ and MIC value of active extracts as anticandidal against C. albicans.

Samples no.	IDZ (mm)	MIC value against *C.albicans* (µg/mL)	Category of MIC Value
18	17.00 ± 1.00^{b}	32.00	Strong
57	9.50 ± 0.00^{e}	32.00	Strong
45	9.17 ± 0.29^{ef}	32.00(P)	Strong
53	18.17 ± 0.29^{a}	64.00	Strong
77	11.50 ± 0.50^{d}	128.00	Moderate
7	7.00 ± 0.00^{i}	128.00(P)	Moderate
43	7.00 ± 0.00^{i}	128.00(P)	Moderate
78	9.83 ± 0.29^{e}	256.00	Moderate
79	8.17 ± 0.29^{fghi}	256.00	Moderate
80	14.00 ± 0.00^{c}	256.00	Moderate
77	11.50 ± 0.50^{d}	128.00	Moderate
6	6.83 ± 0.29^{i}	>256	Weak
9	7.00 ± 0.00^{i}	>256	Weak
11	7.00 ± 0.00^{i}	>256	Weak
12	8.50 ± 0.50^{fg}	>256	Weak
21	11.67 ± 1.53^{d}	>256	Weak
22	7.17 ± 0.29^{ij}	>256	Weak
23	7.17 ± 0.29^{ij}	>256	Weak
27	7.33 ± 0.29^{hij}	>256	Weak
28	7.00 ± 0.00^{i}	>256	Weak
30	8.33 ± 0.29^{fgh}	>256	Weak
32	9.67 ± 0.29^{e}	>256	Weak
33	7.67 ± 0.29^{ghij}	>256	Weak
34	7.83 ± 0.29^{ghij}	>256	Weak
35	8.17 ± 0.29^{fghi}	>256	Weak
36	9.00 ± 0.00^{ef}	>256	Weak
38	7.00 ± 0.00^{i}	>256	Weak
39	7.00 ± 0.00^{i}	>256	Weak
40	7.00 ± 0.00^{i}	>256	Weak
41	7.00 ± 0.00^{i}	>256	Weak
42	7.00 ± 0.00^{i}	>256	Weak
44	9.00 ± 0.00^{ef}	>256	Weak
46	9.17 ± 0.29^{ef}	>256	Weak
48	7.00 ± 0.00^{i}	>256	Weak
51	7.83 ± 0.29^{ghij}	>256	Weak
54	9.67 ± 2.08^{e}	>256	Weak
67	11.00 ± 1.00^{d}	>256	Weak

Note: IZD: Inhibition Zone Diameter. Analysis of variance was performed using Duncan's multiple ranges for IDZ values. Values in each column with distinctive letters are significantly different (P<0.05)

Table 2. IC_{50} and AAI values of active extract as an antioxidant agent

Samples no.	IC50 Value (µg/mL)	AAI Value	Category of AAI Value
43	7.76±0.77a	6.64±0.63a	Very strong
38	7.95±0.73a	6.48±0.63a	Very strong
52	8.53±0.83a	6.04±0.61a	Very strong
28	9.71±0.56a	5.28±0.30b	Very strong
48	12.81±2.29a	4.08±0.69c	Very strong
5	16.34±1.85a	3.16±0.34d	Very strong
24	16.57±1.68a	3.11±0.31d	Very strong
3	25.54±1.37a	2.01±0.10e	Very strong
2	27.60±0.93a	1.86±0.06ef	Strong
47	31.11±4.70a	1.67±0.24efg	Strong
27	31.17±4.14a	1.66±0.21efg	Strong
8	39.41±18.14a	1.61±1.01efg	Strong
63	33.52±4.30a	1.55±0.20efg	Strong
16	37.56±2.68a	1.37±0.10efgh	Strong
80	43.80±10.58a	1.22±030fgh	Strong
42	44.83±4.90a	1.15±0.12fgh	Strong
59	47.49±9.24a	1.10±0.20fgh	Strong
4	50.32±3.65a	1.02±0.08gh	Strong
39	56.02±3.50a	0.92±0.06ghi	Moderate
53	73.41±11.50a	0.71±0.12hij	Moderate
17	202.20±151.35b	0.61±0.74hij	Moderate
58	245.29±46.14b	0.21±0.04ij	Weak
35	393.51±21.28c	0.13±0.01i	Weak

Note: Analysis of variance was performed using Duncan's multiple ranges for IC_{50} and AAI values. Values in each column with distinctive letters differ significantly (P<0.05)

Molecular identification of endophytic fungi

Molecular identification results revealed that several active extracts had anticandidal activity against *C. albicans*, such as extract No. 18 (*Phomopsis* sp. TcTd1Bd-1); No.45 (*Phomopsis* sp. TcBt1Bo-4); No.57 (*Epicoccum* sp. TcDn1Bo-2); Meanwhile, extracts showed very strong active as antioxidants by DPPH radical free scavenging were identified as *Nigrospora* sp. TcBt1Be-2 (extract No. 38); *Phomopsis* sp. TcBt1Bo-2 (extract No. 43) and *Neofusicoccum* sp. TcBt2Bo-1 (extract No.52). Further identification based on molecular approach and neighbor-joining (NJ) phylogenetic analysis method showed that the ITS sequence of *Nigrospora* sp. TcBt1Be-2 has the highest similarity and is nested in the same clade with *N. guilinensis* CGMCC 3.18124 (NR153472). Meanwhile, the ITS sequence of *Neofusicoccum* sp.TcBt2Bo-1 has the highest similarity and is nested in the same clade with *N. grevilleae* CPC 16999 (NR168143) (Table 4, Figure 6).

Table 3. The correlation between TPC, anticandidal and antioxidant activities

Variables	Pearson correlations coefficient (r)	
	Anticandidal MIC against *C. albicans*	Antioxidant AAI value
TPC values	-0.029	0.332*
DZH against *C. albicans*	-0.673*	NT

Note: *= P<0.01

Table 4. Identification of selected active endophytic fungi

Samples no.	Morphological Identification	Molecular Identification *)
18	*Phomopsis* sp. TcTd1Bd-1	ND
45	*Phomopsis* sp. TcBt1Bo-4	ND
57	*Epicoccum* sp. TcDn1Bo-2	ND
38	*Nigrospora* sp. TcBt1Be-2	*Nigrospora guilinensis* CGMCC 3.18124 (Accession no: NR153472) [Similarity: 100.00%; Max score: 953; Total score: 953; Query coverage: 100%; E-value: 0.0; Max identities: 516/516 (100%); Gaps: 0/516 (0%)]
43	*Phomopsis* sp. TcBt1Bo-2	ND
52	*Neofusicoccum* sp.TcBt2Bo-1	*Neofusicoccum grevilleae* CPC 16999 (Accession no: NR168143) [Similarity: 99.14%; Max score: 998; Total score: 998; Query coverage: 100%; E-value: 0.0; Max identities: 581/586 (99%); Gaps: 2/586 (0%)]

Note: *) Closest taxa based on NCBI BLAST (https://blast.ncbi.nlm.nih.gov/). ND: No Data

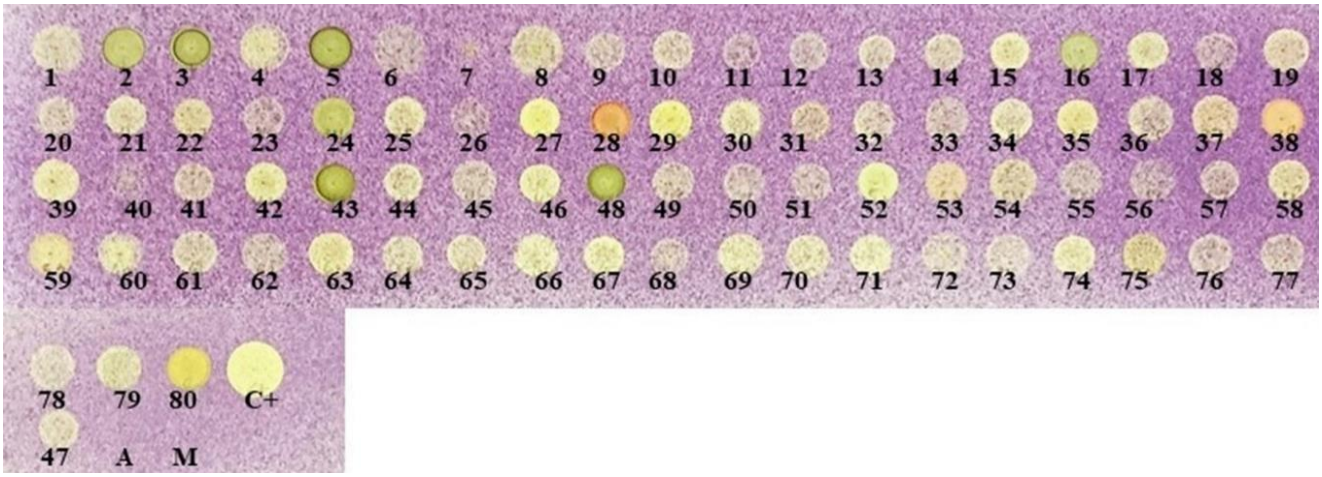

Figure 3. TLC dot-blot assay for antioxidant activity of endophytic fungal extracts. Note: 1-80: 10 µL of 10 mg/mL extract in Acetone; C+: 10 µL of 1 mg/mL quercetin in MeOH; A: 10 µL acetone; M: 10 µL MeOH; after sprayed 0.2 mg/mL DPPH in MeOH

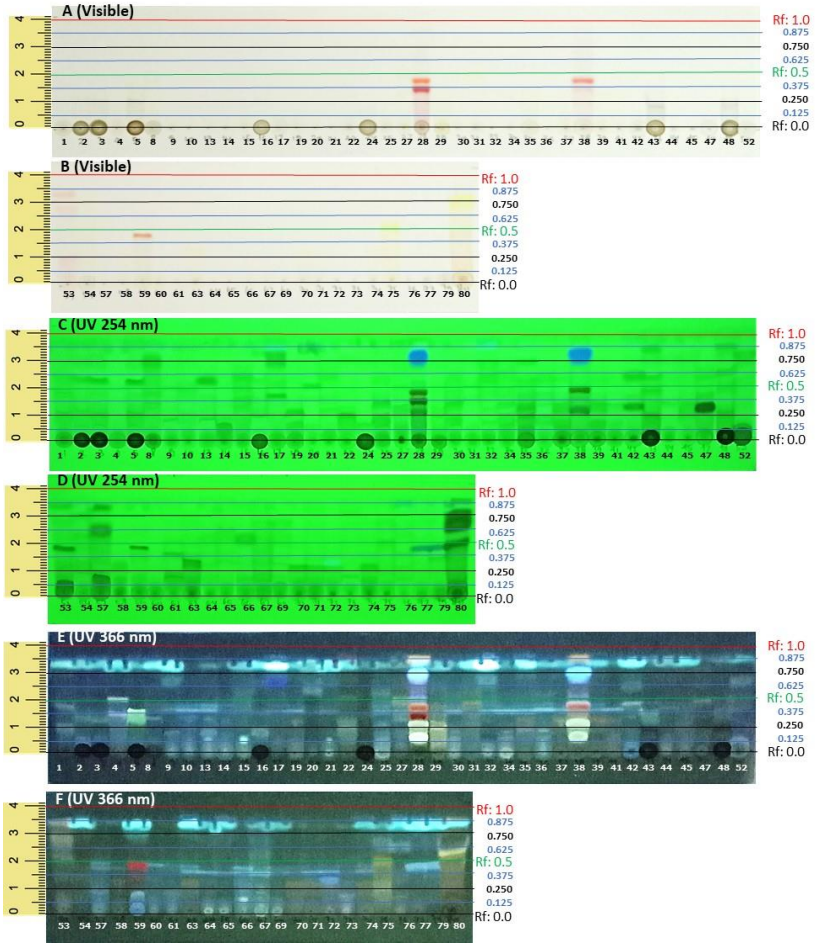

Figure 4. Chromatograms of selected active extracts as an antioxidant agent. Note: Eluent: CH_2Cl_2-methanol (10:1 v/v), viewed under visible light (A-B); under UV light of 254 nm (C-D); under UV light of 366 nm (E-F)

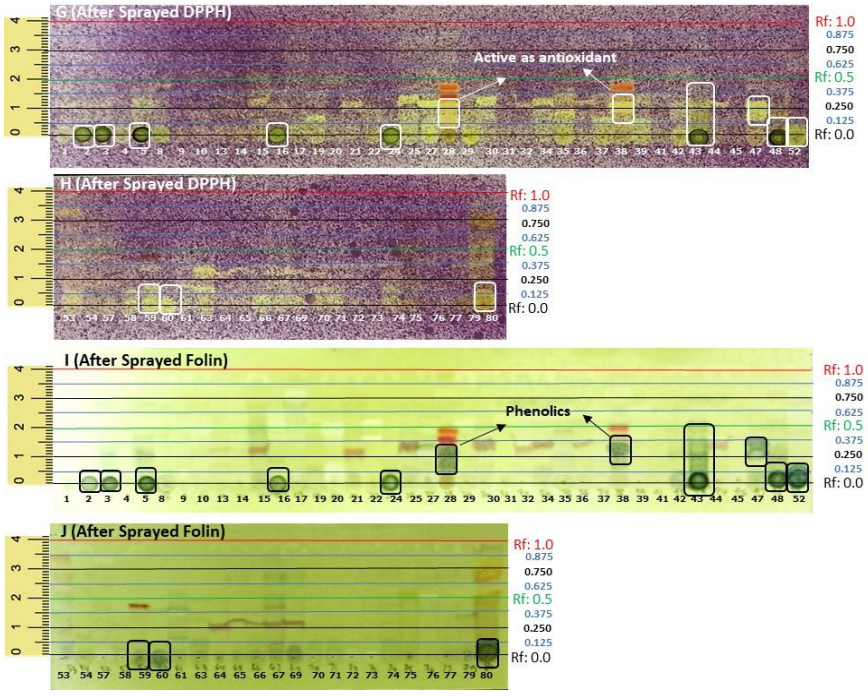

Figure 5. Chromatograms of TLC bioautography toward selected active extracts as an antioxidant agent. Remark: Eluent: CH_2Cl_2-methanol (10:1 v/v), after sprayed 0.2 mg/mL DPPH in MeOH(G-H); after sprayed Folin reagent (I-J)

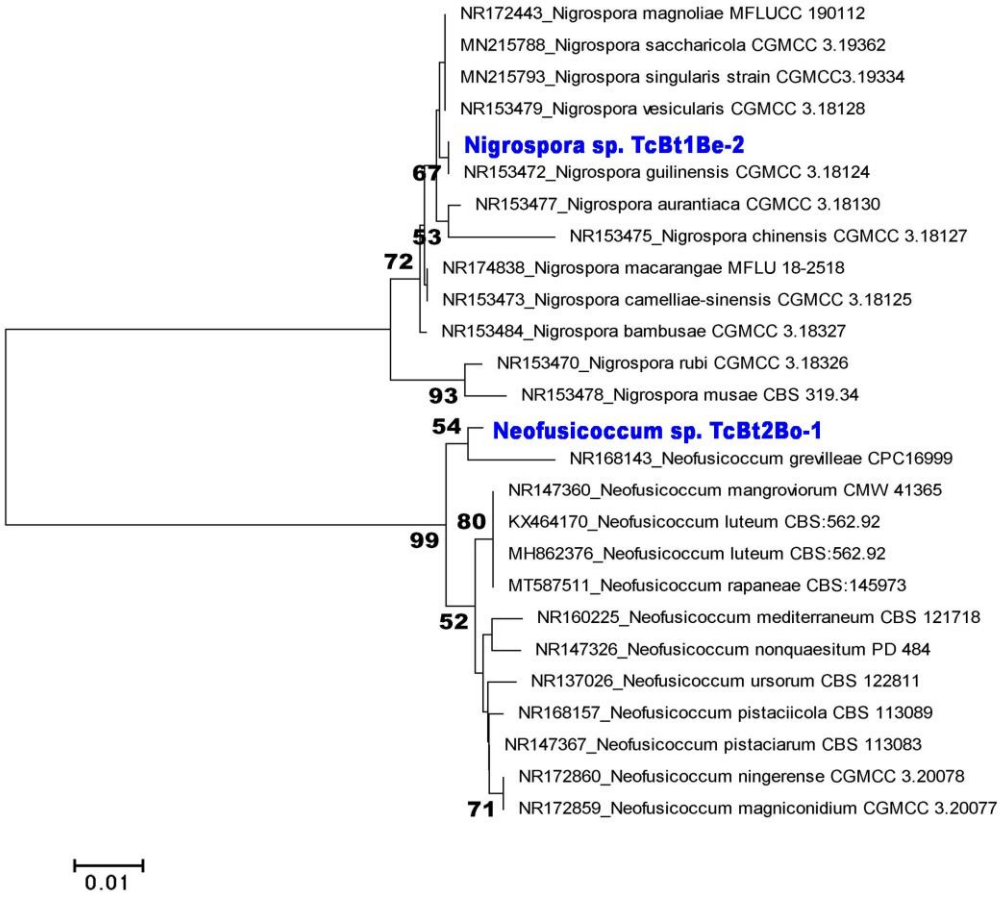

Figure 6. Neighbor-joining tree of selected T. crispa endophytes strain TcBt1Be-2 and TcBt2Bo-1 based on ITS rDNA sequence. Only bootstrap values above 50 are shown

Discussion

The results of the present study revealed that several endophytic fungi possess strong anticandidal activity such as *Phomopsis* sp. TcTd1Bd-1, *Phomopsis* sp. TcBt1Bo-4 and *Epicoccum* sp. TcDn1Bo-2, while *N. guilinensis* TcBt1Be-2., *Phomopsis* sp. TcBt1Bo-2 and *N. grevilleae* TcBt2Bo-1 extracts showed very strong antioxidant activity. Several endophytic fungi showed both anticandidal and antioxidant activities. From previous research, terpenoids from the endophytic fungus *Phomopsis* sp. ED2, isolated from *Orthosiphon stamineus* Benth has anticandidal activity (Yenn et al. 2012). In another study, *Phomopsis asparagi* (Sacc.) Grove from *Calotropis gigantea* (L.) W.T.Aiton also showed anticandidal activity (Nath and Joshi 2017). *Epicoccum* sp. have the capability of producing a wide range of biologically active secondary metabolites. These metabolites have been studied extensively and have demonstrated promising properties such as anticancer, antimicrobial, anti-diabetic, and cytotoxic activities (Fatima et al. 2016). In addition, *Phomopsis* sp. GJJM07 from the medicinal plant *Mesua ferrea* L has antioxidant and anticandidal activities (Jayanthi et al. 2011), *Nigrospora oryzae* (Berk. & Broome) Petch isolated from *Euphorbia hirta* L. has good antioxidant activity in DPPH free radical scavenging (Gautam et al. 2022), and *N. guilinensis* TSU-EFHA009 has strong antifungal activity against *Cryptococcus neoformans* (San Felice) Vuill. as well as good antioxidant activity with an IC_{50} value of 30 µg/mL (Supaphon and Preedanon 2019). On the other hand, *Neofusicoccum ribis* (Slippers, Crous & M.J.Wingf.) Crous, Slippers & A.J.L.Phillips from *Bouea macrophylla* Griff. has activity as an inhibitor of AChE and *β*-glucosidase enzymes, antibacterial and antioxidant (Anindyawati and Praptiwi 2019), however, there is no information regarding antioxidants from *N. grevilleae*.

The results of the anticandidal activity assay showed that several extracts have strong anticandidal (MIC<100 µg/mL) against *C. albicans*, as well as antioxidant activity assay, also showed that several extracts have very strong antioxidants (AAI>2) as scavenging of DPPH-free radicals. Total phenolic content (TPC) is known to contribute to the antioxidant activity of natural extracts (Muflihah et al. 2021). An isolated compound from *Phomopsis* sp. TcBt1Bo-6 (sample No.47) is (+)-epoxydone with *Rf* 0.250-0.375 and a dark spot under UV of 256 nm. It has strong antibacterial activities against *E. coli* (Fathoni et al. 2022c). This compound also has anticandidal activity against *C. albicans* with a MIC value of 50 partial (moderate), and antioxidant activity with IC_{50} >25 µg/mL or AAI value of <1.23 (weak antioxidant activity) (unpublished data). In addition, a phenolic compound from

the fungus *Xylaria* sp. DAP KRI-5 such as phloroglucinol has antibacterial activity (Fathoni et al. 2013). It also has strong antioxidant activity against DPPH free radicals with IC_{50} of 4.70±0.38 µg/mL and AAI value of 6.57±0.51 (unpublished data).

In this study, the TPC variable had a weak positive correlation with AAI value (r-value 0.332, P <0.01) and a weak negative with MIC value (r-value -0.029). Meanwhile, the value of the inhibition zone diameter (anticandidal) had a moderate negative correlation with the MIC value of *C. albicans* (r-value -0.673, P<0.01). The previous study indicated a strong correlation between total phenolic with antioxidant capacity (Shrestha and Dhillion 2006). The bioactive compounds within the extract may have a synergistic action that causes vigorous activity for free radical scavenging as antioxidant agents (Praptiwi et al. 2018).

This study concluded that the extract of fungal endophytes from *T. crispa* showed anticandidal and antioxidant activity. This report indicates that endophytic fungi isolated from *T. crispa* may have potential in the future as drug discovery as natural anticandidal and antioxidant.

ACKNOWLEDGEMENTS

The authors would like to express thanks to Saintek-BRIN Scholarship for giving the Doctoral scholarship to the first author and Kemendikbudristek RI (*Kementerian Pendidikan, Kebudayaan, Riset, dan Teknologi*) for the research grant of PDD 2022 to fund this research and publication.

REFERENCES

Agusta A, Nurkanto A, Fathoni A, Jamal Y, Praptiwi P. 2013. 3-Acetyl-2,5,7-Trihydroxy-1,4-Naphtalenedione, an antimicrobial metabolite from the culture of endophytic fungus Coelomycetes TCBP4 from *Tinospora crispa*. Media Penelitian dan Pengembangan Kesehatan 23 (3): 95-101. DOI: 10.22435/mpk.v23i3.3278.95-101.

Ahmad W, Jantan I, Bukhari SNA. 2016. *Tinospora crispa* (L.) Hook. f. & Thomson: A review of its ethnobotanical, phytochemical, and pharmacological aspects. Front Pharmacol 7: 1-19. DOI: 10.3389/fphar.2016.00059.

Alam B, Lǐ J, Gě Q, Khan MA, Gōng J, Mehmood S, Yuán Y, Gǒng W. 2021. Endophytic fungi: From symbiosis to secondary metabolite communications or vice versa? Front Plant Sci 12: 791033. DOI: 10.3389/fpls.2021.791033.

Anindyawati T, Praptiwi. 2019. A study of endophytic fungi *Neofusicoccum ribis* from gandaria (*Bouea macrophylla* Griffith) as enzyme inhibitor, antibacterial, and antioxidant. Appl Biol Biotechnol 7 (1): 48-53. DOI: 10.7324/JABB.2019.70109.

Atasoy N, Yücel UM. 2021. Antioxidants from Plant Sources and Free Radicals. In: Ahmad R (eds.). Reactive Oxygen species. IntechOpen, London. DOI: 10.5772/intechopen.100350.

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal 6 (2): 71-79. DOI: 10.1016/j.jpha.2015.11.005.

Chutulo EC, Chalannavar RK. 2018. Endophytic mycoflora and their bioactive compounds from *Azadirachta indica*: A comprehensive review. J Fungi 4 (2): 42. DOI: 10.3390/jof4020042.

Fathoni A, Hudiyono S, Budianto E, Cahyana AH, Agusta A. 2021. Metabolite detection and antibacterial activity of fungal endophytic extracts isolated from *brotowali* (*Tinospora crispa*) plants using TLC-Bioautography Assay. IOP Con Ser: Mater Sci Eng 1011: 12041. DOI: 10.1088/1757-899x/1011/1/012041.

Fathoni A, Ilyas M, Cahyana AH, Agusta A. 2013. Screening and isolation of antibacterial active metabolite from the culture of endophytic fungi from Albertisia Papuana Becc. Berita Biologi 12 (3): 307-314. [Indonesian]

Fathoni A, Ilyas M, Praptiwi, Wulansari D, Agusta A. 2022a. Antibacterial and antioxidant activities of fungal endophytes isolated from medicinal plants in Simeulue Island, Aceh. HAYATI J Biosci 29 (6): 720-732. DOI: 10.4308/hjb.29.6.720-732.

Fathoni A, Hudiyono S, Budianto E, Cahyana AH, Ilyas M, Agusta A. 2022b. Evaluation of antibacterial activity, total phenolic and flavonoid contents of extracts of endophytic fungi associated with *Tinospora crispa* (L.) Hook. f. & Thomson. J Adv Sci Eng Inf Technol 12 (5): 1728-1735. DOI: 10.18517/ijaseit.12.5.14816.

Fathoni A, Hudiyono S, Cahyana, AH, Nurkanto A, Agusta A. 2022c. (+)-epoxydone: A major secondary metabolite as antibacterial agent from Phomopsis sp. TcBt1Bo-6 isolated from stem of *brotowali* plant. Rasayan J Chem 15 (4): 2210-2217. DOI: 10.31788/RJC.2022.1546982.

Fatima N, Ismail T, Muhammad SA, Jadoon M, Ahmed S, Azhar S, Mumtaz A. 2016. *Epicoccum* sp., an emerging source of unique bioactive metabolites. Acta Pol Pharm 73 (1): 13-21.

Gautam VS, Singh A, Kumari P, Nishad JH, Kumar J, Yadav M, Bharti R, Prajapati P, Kharwar RN. 2022. Phenolic and flavonoid contents and antioxidant activity of an endophytic fungus *Nigrospora sphaerica* (EHL2), inhabiting the medicinal plant *Euphorbia hirta* (dudhi) L. Arch Microbiol 204 (2): 1-13. DOI: 10.1007/s00203-021-02650-7.

Gouda S, Das G, Sen SK, Shin HS, Patra JK. 2016. Endophytes: A treasure house of bioactive compounds of medicinal importance. Front Microbiol 7: 1-8. DOI: 10.3389/fmicb.2016.01538.

Harper JK, Arif AM, Ford EJ, Strobel GA, Porco JA, Tomer DP, Oneill KL, Heider EM, Grant DM. 2003. Pestacin: a 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. Tetrahedron 59 (14): 2471-2476. DOI: 10.1016/S0040-4020(03)00255-2.

Ilyas M, Praptiwi, Wulansari D, Fathoni A, Agusta A. 2019. An assemblages of fungal endophytes isolated from medicinal plants collected from Toba and Samosir, North Sumatra. IOP Conf Ser Earth Environ Sci 308 (1): 012070. DOI: 10.1088/1755-1315/308/1/012070.

Jayanthi G, Kamalraj S, Karthikeyan K, Muthumary J. 2011. Antimicrobial and antioxidant activity of the endophytic fungus *Phomopsis* sp. GJJM07 isolated from *Mesua ferrea*. Intl J Curr Sci 20: 85-90.

Kandel SL, Joubert PM, Doty SL. 2017. Bacterial endophyte colonization and distribution within plants. Microorganisms 5 (4): 9-11. DOI: 10.3390/microorganisms5040077.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874. DOI: 10.1093/molbev/msw054

Li S, Chen G, Zhang C, Wu M, Wu S, Liu Q. 2014. Research progress of natural antioxidants in foods for the treatment of diseases. Food Sci Hum Wellness 3 (3-4): 110-116. DOI: 10.1016/j.fshw.2014.11.002.

Lorena BB, Javiera O, Jean Franco C. 2021. Chapter 1 - Facultative fungal endophytes and their potential for the development of sustainable agriculture. In: Kumar A, Droby S (eds.). Microbial Management of Plant Stresses. Woodhead Publishing, Cambridge. DOI: 10.1016/B978-0-323-85193-0.00014-0.

Muflihah YM, Gollavelli G, Ling YC. 2021. Correlation sudy of antioxidant activity with phenolic and flavonoid compounds in 12 Indonesian indigenous herbs. Antioxidants 10 (10): 1530. DOI: 10.3390/antiox10101530.

Napitupulu TP, Ilyas M, Kanti A, Sudiana IM. 2019. In vitro evaluation of *Trichoderma harzianum* strains for the control of *Fusarium oxysporum* f.sp. *cubense*. Plant Pathol Quarantine 9 (1): 152-159. DOI: 10.5943/ppq/9/1/13.

Nath A, Joshi S. 2017. Anti-candidal effect of endophytic fungi isolated from *Calotropis gigantea*. Rev Biol Trop 65 (4): 1437. DOI: 10.15517/rbt.v65i4.26269.

Praptiwi, Raunsai M, Wulansari D, Fathoni A, Agusta A. 2018. Antibacterial and antioxidant activities of endophytic fungi extracts of medicinal plants from Central Sulawesi. J Appl Pharm Sci 8 (8): 69-74. DOI: 10.7324/JAPS.2018.8811.

Rana KL, Kour D, Kaur T, Devi R, Yadav AN, Yadav N, Dhaliwal HS, Saxena AK. 2020. Endophytic microbes: biodiversity, plant growth-

promoting mechanisms and potential applications for agricultural sustainability. Antonie van Leeuwenhoek 113 (8): 1075-1107. DOI: 10.1007/s10482-020-01429-y.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425. DOI: 10.1093/oxfordjournals.molbev.a040454.

Shrestha PM, Dhillion SS. 2006. Diversity and traditional knowledge concerning wild food species in a locally managed forest in Nepal. Agrofor Syst 66 (1): 55-63. DOI: 10.1007/s10457-005-6642-4.

Supaphon P, Preedanon S. 2019. Antimicrobial and antioxidant activities of endophytic fungi extracts isolated from *Carissa carandas*. Afr J Microbiol Res 13 (27): 464-473. DOI: 10.5897/ajmr2019.9164.

Suresha BS, Srinivasan K. 2013. Antioxidant properties of fungal metabolite nigerloxin in vitro. Appl Biochem Microbiol 49 (6): 587-591. DOI: 10.1134/S0003683813060173.

Wen J, Okyere SK, Wang S, Wang J, Xie L, Ran Y, Hu Y. 2016. Endophytic fungi: an effective alternative source of plant-derived bioactive compounds for pharmacological studies. J Fungi 8 (2): 1-45. DOI: 10.3390/jof8020205.

White TJ, Bruns TD, Lee SB, Taylor JW. 1990. Amplification and direct sequencing of fungal RNA genes for phylogenetics. PCR protocols, 315-322. Academic Press, San Diego. DOI: 10.1016/B978-0-12-372180-8.50042-1.

Wulansari D, Praptiwi, Julistiono H, Nurkanto A, Agusta A. 2016. Antifungal activity of (+)-2,2'-Epicytoskyrin A and its membrane-disruptive action. Makara J Sci 20 (4): 160-166. DOI: 10.7454/mss.v20i4.6703.

Yenn TW, Lee CC, Ibrahim D, Zakaria L. 2012. Enhancement of anti-candidal activity of endophytic fungus *Phomopsis* sp. ED2, isolated from *Orthosiphon stamineus* Benth, by incorporation of host plant extract in culture medium. J Microbiol 50 (4): 581-585. DOI: 10.1007/s12275-012-2083-8.

Zida A, Bamba S, Yacouba A, Ouedraogo-Traore R, Guiguemdé RT. 2017. Anti-Candida albicans natural products, sources of new antifungal drugs: A review. J Mycol Med 27 (1): 1-19. DOI: 10.1016/j.mycmed.2016.10.002.