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Metabolites produced by endophytic fungi isolated from *Hornstedtia scyphifera* (J. Koenig) Steud. and their antioxidant activities

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Abstract

Importance of the work: Antioxidant-producing fungi from medicinal plants are still underexplored, especially those originating from wild and uncommon species of the Zingiberaceae.

Objectives: To designate the potential fungal strains exhibiting prominent antioxidant activities through *in vitro* assay and to identify the putative compounds.

Materials & Methods: Endophytic fungal isolates from the rhizome part of *H. scyphifera* were identified to the species level based on their internal transcribed spacer (ITS)-rDNA molecular regions. Screening of potential isolates was based on their antibacterial activities against human pathogenic bacteria. The metabolites obtained from batch fermentation of potential strains were evaluated for their antioxidant activities using 2,2-diphenyl-1-picryl-hydrazyl-hydrate, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) and ferric reducing antioxidant power assays. High performance liquid chromatography with diode array detection was used to determine the active compounds.

Results: Two fungal isolates, namely H4 and H7, later identified as *Xylaria* sp. and *Pyrrhoderma* sp., respectively, using molecular regions (ITS-rDNA), were selected because they showed strong antagonism against human pathogenic bacteria. The highest antioxidant activity was achieved through the use of MeOH (methanol) > EtOAc (ethyl acetate) > C6 (hexane) by *Xylaria* sp. H4. Crude EtOAc extract of *Xylaria* sp. H4 yielded two compounds (chlorogenic acid [CGA] and caffeic acid), while the MeOH extract yielded five compounds (catechin, CGA, caffeic acid, gallic acid and luteolin). Crude EtOAc extract of *Pyrrhoderma* sp. H7 yielded no compounds, while the MeOH extract yielded the same number of compounds as *Xylaria* sp. H4.

Main finding: The results provided new information for endophytic fungi that can be exploited as a source of antioxidant compounds and possibly other medicinal aspects from *Hornstedtia scyphifera*.

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Introduction

Indonesia is a hotspot for a variety of medicinal plants that are intensively utilized and formulated to produce herbal medicine, popularly known as *jamu*, one of which is derived from the Zingiberaceae family (Sholikhah et al., 2016). The majority of Zingiberaceae species are used as a source of bioactive agents and raw materials for traditional medicine by some indigenous tribes in Indonesia (Fernando and Nursyahra, 2008; Hartanto et al., 2014; Putra et al., 2015). Laboratory investigation of Zingiberaceae metabolites has revealed their bioactive potential, such as anti-allergic, anti-diabetic, anti-inflammatory, antimicrobial, antioxidant and anti-tumor properties, which provide physiological balance to the human body (Habsah et al., 2000; Ho, 2011; Chan and Wong, 2015; Juwita et al., 2018). The discovery of sustainable sources of bioactive metabolites may be accelerated through the exploration of plant-associative microorganisms, known as microbial endophytes (Strobel, 2018).

Due to their ability to adapt to environmental changes, especially growth conditions, endophytic microorganisms, particularly the fungal endophytes community, have unique characteristics in terms of metabolite production (Radić and Štrukelj, 2012). The notion of "One Strain, Many Compounds" (OSMAC) is used to modify the fungal physiology in order to synthesize a variety of metabolites during fermentation on various media (Abdelwahab et al., 2018). The majority of the metabolites possessing antibacterial, antioxidant and anticancer properties are synthesized by fungal endophytes (Kaul et al., 2012; Deshmukh et al., 2015). Exploration, elaboration and justification of the desired traits by endophytic fungi are continuously being promoted in the biomedical field to deal with pathogenic infections and other health problems (Radić and Štrukelj, 2012). The potential of superior endophytic fungal isolates may be explored by studying various species of the Zingiberaceae (Chakraborty et al., 2019).

Endophytic fungi from the Zingiberaceae of *Hedychium coronarium, H. flavescens, and Z. officinale* produce extracellular enzymes, having good prospects for industrial development (Uzma et al., 2016). Endophytic fungal isolates from Indonesian red ginger produced antagonistic activities against *Fusarium oxysporum* that has been useful for biocontrol of agricultural diseases (Ginting et al., 2013). Hexane extract from *F. oxysporum* filtrate isolated from the rhizome of Indian *Zingiber zerumbet* secreted potential antioxidant compounds with COX II-inhibitory activity, which can be formulated as biomedicines (Nongalleima et al., 2013). In addition, an ethyl acetate fraction of *Arthrinium* sp. MFLUCC16-1053, an endophytic fungus from *Z. cassumunar*, was reported to have potential bioactive activity (Pansanit et al., 2018).

Exploration of endophytic fungi extracted from the Zingiberaceae has been initiated in the North Sumatra region, where a number of endophytic fungal isolates have been reported and are associated with several genera of the Zingiberaceae, including *Amomum, Elettaria, Hedychium* and *Globba* (Lutfia et al., 2019a, b, c; Lutfia et al., 2020; Munir et al., 2020). Some uncommon zingiberaceous species still hold the possibility of finding novel assemblages of fungal endophytes, supporting the relevance of studying their chemical diversity. The current study evaluated the antioxidant activities of secondary metabolites extracted from the batch-fermentation of fungal endophytes isolated from a zingiberaceous species, *Hornstedia scyphifera* (J. Koenig) Steud. Determination of the chemical constituents of the extracts was performed using high performance liquid chromatography with diode array detection (HPLC-DAD) and comparing the results with a set of standard compounds for phenolics and flavonoids.

Materials and Methods

Plant materials

Hornstedtia scyphifera (J. Koenig.) Steud. rhizome was collected from Sibayak Forest, Deli Serdang District, North Sumatra, Indonesia (Fig. 1). Disease-free *H. scyphifera* plants

Fig. 1 *Hornstedtia scyphifera* (J. Koenig) in Sibayak Forest, North Sumatra

were collected and placed in sterile plastic bags and transported to the laboratory. The plant samples were kept refrigerated until further processing. A duplicate sample was sent to the Herbarium Medanese, Universitas Sumatera Utara for species identification. Endophytic fungal isolation was completed within 48 h of sample processing.

Isolation of endophytic fungi

Isolation of endophytic fungi was carried out in accordance with the surface sterilization method described by Lutfia et al. (2021). Rhizomes were gently washed under running tap water before being chopped into 10 cm segments. Surface sterilization was performed using 75% ethanol (EtOH) for 2 min, 5.3% sodium hypochlorite (NaOCl) for 5 min, and 75% of EtOH for 1 min, followed by three 1 min rinses with sterile distilled water. The sterilized rhizome segments were chopped into 1–2-cm fragments and placed on Petri dishes (90 mm diameter) with potato dextrose agar (PDA) enriched with 0.01% chloramphenicol (weight per volume, w/v). A sample (1 mL) of the final rinsing solution was spread on PDA medium to verify the surface sterilization technique. The plates were kept at room temperature (25 ± 3 °C) and were checked daily for the establishment of colonies from the rhizome fragments. Any individual colony that grew was carefully chosen and transplanted to new PDA plates. For maintenance and subsequent research, stock cultures were created by sub-culturing fungal colonies. Each isolate was classified based on colony features and given a code that was used throughout the investigation.

Antagonistic test of endophytic fungi against human pathogenic bacteria

Each isolate was tested for its ability to inhibit the growth of human pathogenic bacteria, *Staphylococcus aureus* ATCC® 29213™, methicilin-resistant *S. aureus* ATCC® 43300™, *Escherichia coli* ATCC® 25922™ and *Klebsiella pneumoniae* ATCC® 700603 using a modified dual culture method (Lutfia et al., 2019a). Three 6 mm mycelial plugs were cut from the active-growing colony and placed on top of a PDA plate that had previously been seeded with 1–2 × 10^8 colony forming units/mL of pathogen suspensions and supplemented with 1% yeast extract (w/v). An antibiotic plug containing 1% of chloramphenicol (w/v) in PDA and an agar plug of PDA medium were used as the positive and negative controls, respectively. The plates were incubated for two days at 37 °C. Clear zones around mycelial plugs indicated the presence of antagonistic actions (Fig. S1); colony size was measured using digital calipers in millimeters based on the diameter.

Molecular identification of endophytic fungi

Based on the sequence of the internal transcribed spacer (ITS) rDNA, each fungal isolate was identified to the genus level and, if possible, to the species level. A Wizard® Genomic DNA Purification Kit (USA) was used to extract DNA, which was done according to the manufacturer's instructions. ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') universal primers for fungi were used in the polymerase chain reaction (PCR). The reaction was made up of 12 µL of nuclease-free water, 20 µL of GoTaq Green Master Mix (@200 µM of dATP, dGTP, dCTP, dTTP), 2 µL of each primer (2.5 pmol) and 4 µL of DNA template solution (50 ng) in a microcentrifuge tube with a final volume of 40 µL. The PCR reaction was perfomed in the order: 95 °C (3 min), 35 cycles of 95 °C (45 s), 55 °C (45 s), 72 °C (45 s) and 72 °C (7 min). The PCR products were observed on 1% (w/v) agarose gels and sent to Macrogen, Inc. Singapore for sequencing. MEGA X's MUSCLE feature was used to build multiple sequence alignments (Kumar et al., 2018). After retrieving the BLAST findings (http://blast.ncbi.nlm.nih.gov/), the ITS sequences were built into a dendrogram using the neighbor-joining clustering method; the results were analyzed and a phylogenetic tree was built utilizing each isolate's ITS sequence and a representative database based on the BLAST search results. The Kimura-2 model, gamma distribution and bootstrapping with as many as 10,000 replicates were used to analyze kinship based on genetic distance.

Batch fermentation and extraction of metabolites

The fermentation condition was created in accordance with the standard protocol followed for the study of metabolites in endophytic fungi through solvent extraction (Kumar and Kaushik, 2013). Briefly, five agar plugs of potential fungal isolates were inoculated into 250 mL of yeast malt broth (Wickerham medium) for 4 wk in the dark at 25 ± 3 °C under static conditions. After 24 d of incubation, 250 mL of ethyl acetate (EtOAc) was added to the broth, mixed and left overnight. The immersed fungal culture was blended for 15 min and passed through Whatman filter paper. The filtrate was collected

and the residual phase was partitioned with 90% methanol (MeOH) and 100% n-hexane (C6). The solvent extracts were dried in a vacuum rotary evaporator and were subjected to further assay.

2,2-Diphenyl-1-picryl-hydrazyl-hydrate radical scavenging assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a free radical that produces a violet solution in EtOH and becomes colorless when it reacts with an antioxidant molecule, with the antioxidant assay method based on a slight modification from Makris et al. (2003). Briefly, the DPPH reagent was freshly prepared (0.04 mg/mL) in EtOH and mixed with 70 µL of samples with varying concentrations and incubated at room temperature for 10 min. The radical scavenging activity of the tested samples against DPPH was determined by measuring the ultraviolet absorbance (A_{517}) with MeOH as a blank solution using a microplate reader. The scavenging ability was calculated using Equation 1:

$$\text{DPPH radical scavengind activity (\%)} = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100 \quad (1)$$

Ascorbic acid was used as the standard antioxidant and was tested in the same manner as the samples. The half maximal inhibitory concentration (IC_{50}, measured in parts per million, ppm) value was calculated using Microsoft Excel 2019 (USA) by plotting the concentration of samples (in parts per million) and the scavenging activity (as a percentage) to generate an equation for the value.

Determination of total antioxidant capacity

ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] is a blue-green free radical that is stable at room temperature and is commonly used as an indicator of antioxidant together with Trolox in what is commonly known as Trolox equivalent antioxidant capacity (TEAC) assay (Shan et al., 2007). The antioxidant assay was performed based on a slight modification from Avanti et al. (2021). Briefly, 15 µL of sample were mixed with 180 µL of ABTS solution and incubated at 37 °C in the dark for 30 min. The antioxidant capacity was expressed as the decrease in percentage of absorbance (A_{734}) that was calculated using Equation 2:

$$\text{ABTS radical inhibitory activity (\%)} = [(1-Abs_{sample})/Abs_{blank}] \times 100 \quad (2)$$

where the absorbance of the blank sample (Abs_{blank}) was read in MeOH solution at t = 0 min, while the absorbance of the tested sample (Abs_{sample}) was recorded at t = 30 min. The IC_{50} value was determined in the same manner as described above in the DPPH assay.

Determination of reducing power

The FRAP (ferric reducing antioxidant power) assay is an antioxidant assay that measures the reduction of the ferric ion (Fe^{3+})-ligand complex to the ferrous (Fe^{2+}) complex by antioxidant molecules, as indicated from the intense blue colored solution; the FRAP assay was performed based on a slight modification from Pulido et al. (2000). Briefly, 70 µL of sample were mixed with 200 µL of FRAP solution and incubated at 37 °C for 4 min. The results were calibrated from the absorbance reading using $FeSO_4$ (25–1,100 µM) and MeOH as a blank. The reducing power of antioxidant-bearing samples was determined based on the fresh-weight basis or micromoles of Fe(II) per liter of sample at A_{593}.

Identification of antioxidant compounds

The extracts showing potential antioxidant activities were analyzed for bioactive antioxidant compounds based on an HPLC-DAD technique (Pan et al., 2017). Identification of individual compounds was based on the retention times of reference standards (apigenin, caffeine, catechin, chlorogenic acid, cyanidin-3-O-glycoside, ferulic acid, gallic acid (GA), icariin, luteolin, phlorizin, rosmarinic acid and rutin). An Agilent 1100 series (USA) unit with a Torrance C_{18} 110A column 110A column (250 nm × 4.60 nm, 5 µm, USA) was used as the HPLC system. Five µL of each sample were injected into the instrument for separation using mobile phases consisting of acetonitrile (A) and 0.2% acetic acid aqueous solution (B) at a flow rate of 1.0 mL/min at 30 °C and samples (5 L each) were applied to the HPLC-DAD system for separation using the following gradient elution program: 0–12 min with a linear gradient of 5–40% A; 12–18 min with a linear gradient of 40–50% A; 18–22 min with a linear gradient of 50–65% A; 22–25 min with a linear gradient of 65–95% A; 25–30 min with a linear gradient of 95% A; and 30–35 min with a linear gradient of 95–5% A. The detection was carried out at 280 nm.

Statistical analysis

All the experiments were conducted in triplicate. Mean ± SD values were determined using Minitab version 17.0 (USA). The results of antagonistic and antioxidant activities were expressed as mean ± SD values. One-way analysis of variance (ANOVA) and Student's t test were used to determine statistical differences among antagonistic activities. One-way ANOVA followed by Tukey's multiple comparison test were used to determine statistical differences among antioxidant activities.

Results

Eight strains of morphologically distinct fungal morphotypes were isolated from the *H. scyphifera* rhizome. All endophytic fungal isolates were extracted for genomic DNA and gene amplification using PCR in the ITS-rDNA region, based on ITS1 and ITS4 as universal primers for fungal identification. The ITS sequences from each isolate were analyzed using BLAST by referring to the database targeted location project for ITS-rRNA. The results of identification based on the highest maximum score indicated that each isolate had a different identity (Table 1). Antagonistic testing was performed against representatives of human pathogenic bacteria (*S. aureus*, MRSA, *E. coli*, *K. pneumoniae*). The results (Table 2) showed that strains H4 and H7 produced the highest inhibition towards tested pathogens. Thus, the amplified ITS1-5.8S rDNA-ITS2 regions of these two potential isolates were submitted to GenBank (H4: OP788036.1, H7: OP788037.1) and selected for further analysis. The phylogenetic relations of the isolates were compared with other fungal species retrieved from the NCBI GenBank database. Isolate H4 was ascribed to a xylariaceous fungus or *Xylaria* sp. (Xylariaceae: Ascomycota) while isolate H7 was ascertained as *Pyrrhoderma* sp. (Hymenochaetaceae: Basidiomycota).

Metabolites obtained from the two isolates, *Xylaria* sp. H4 and *Pyrrhoderma* sp. H7, were subjected to antioxidant assay based on their highest bioactivity against human pathogenic bacteria. The antioxidant extracts from each isolate displayed different antioxidant capacities in terms of IC_{50} (DPPH, ABTS) and reducing power (FRAP), as shown in Fig. 3. In the DPPH assay, the antioxidant capacity (expressed as an IC_{50} value) ranged widely from 48.41 ± 11.82 ppm (MeOH) to 190.1 ± 33.8 ppm (hexane) for *Xylaria* sp. H4 and from 99.4 ± 17.8 ppm (MeOH) to 213. 1 ± 32.5 ppm (hexane) for *Pyrrhoderma* sp. H7. The antioxidant activity was significant for the MeOH extract of *Xylaria* sp. H4 ($F = 32.38$, $p = 0.001$) and *Pyrrhoderma* sp. H7 ($F = 15.55$, $p = 0.004$) among the tested extracts and was significantly distinct among fungal strains ($t = -4.14$, $p = 0.026$).

Table 1 BLAST search results of highest hits of identified internal transcribed spacer-rDNA fungal species

Isolate	Species	Accession number	Query cover	E-value	Percent Identity
H1	*Tolypocladium album* CBS 869.73	NR_155018	100%	0.0	97.65%
H2	*Memnoniella longistipitata* ATCC 22699	NR_155538	100%	0.0	95.52%
H3	*Gliomastix polychroma* CBS 181.27	NR_119408	100%	0.0	98.97%
H4	*Xylaria longissima* IRAN 2268C	NR_147567	100%	0.0	88.87%
H5	*Ganoderma meredithae* CBS 271.88	NR_164435	100%	0.0	94.33%
H6	*Annulohypoxylon thailandicum* MFLUCC 13-0118	NR_153529	77%	0.0	99.85%
H7	*Pyrrhoderma hainanense* IFP 019153	NR_158943	98%	1e-161	85.71%
H8	*Rostrohypoxylon terebratum* CBS 119137	NR_137677	45%	1e-128	87.65%

E-value = Expect value

Table 2 Antibacterial activity of endophytic fungi from *Hornstedtia scyphifera*

Species	mean diameter ± SD of inhibitory zone (mm))			
	S. aureus	MRSA	*E. coli*	*K. pneumoniae*
Tolypocladium album H1	-	-	-	-
Memnoniella sp. H2	-	-	-	-
Gliomastix polychroma H3	-	-	-	-
Xylaria sp. H4	20.03 ± 1.86 a	10.58 ± 1.72 a	15.37 ± 2.35 a	11.20 ± 1.21 a
Ganoderma sp. H5	12.33 ± 1.56 b	9.64 ± 1.80 a	-	-
Annulohypoxylon thailandicum H6	-	-	-	-
Pyrrhoderma sp. H7	22.23 ± 1.12 a	20.03 ± 2.11 b	17.20 ± 0.70 a	13.33 ± 1.53 a
Xylariaceae fungus H8	9.00 ± 0.60 b	-	-	-

MRSA = Methicilin-resistant *S. aureus*

Mean±SD in each column superscripted with different lowercase letters are significantly ($p < 0.05$) different.

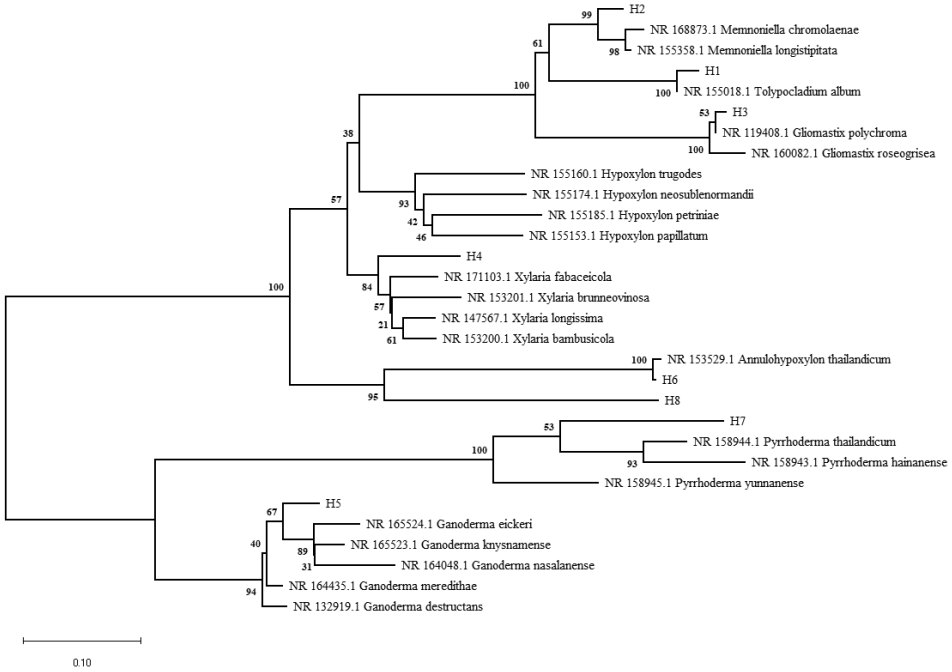

Fig. 2 Phylogenetic inference of endophytic fungal isolates from *Hornstedtia scyphifera* based on neighbor-joining method with 10,000× bootstrapping

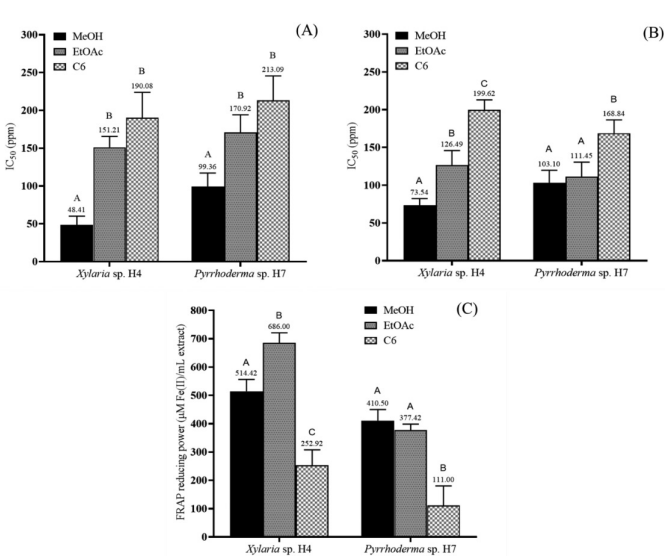

Fig. 3 Antioxidant capacity of methanol (MeOH), ethyl acetate (EtOAc) and hexane (C6) extracts from *Xylaria* sp. H4 and *Pyrrhoderma* sp. H7: (A) 2,2-diphenyl-1-picryl-hydrazyl-hydrate; (B) 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate); (C) ferric reducing antioxidant power (FRAP), where IC_{50} is the half maximal inhibitory concentration, ppm is parts per million and different letters above error bars indicate significant ($p < 0.05$) differences within each species.

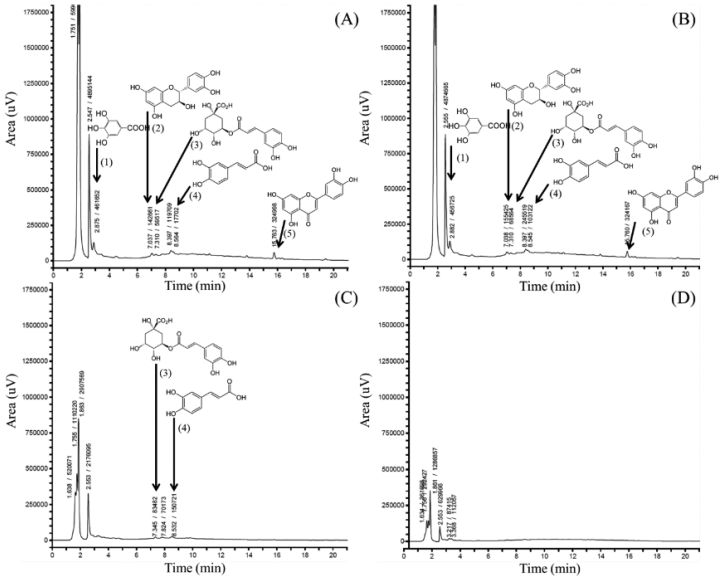

Fig. 4 Antioxidant compounds detected during analysis as depicted from retention times for fungal extracts: (A) methanol (MeOH)-*Xylaria* sp. H4; (B) MeOH-*Pyrrhoderma* sp. H7; (C) ethyl acetate (EtOAc)-*Xylaria* sp. H4; (D) EtOAc-*Pyrrhoderma* sp. H7, where standard antioxidants are: (1) gallic acid, (2) catechin, (3) chlorogenic acid, (4) caffeic acid and (5) luteolin

In the ABTS assay, the IC_{50} value for *Xylaria* sp. H4 ranged between 73.54 ± 8.72 ppm (MeOH) and 199.62 ± 13.43 ppm (hexane) while for *Pyrrhoderma* sp. H7 it was between 103.10 ± 16.59 ppm (MeOH) and 168.8 ± 17.6 ppm (hexane). There was a significant difference among tested extracts in *Xylaria* sp. H4 ($F = 57.08$, $p = 0.000$) but not in *Pyrrhoderma* sp. H7 ($F = 12.11$, $p = 0.008$). The ABTS radical scavenging activity was not significantly different among fungal strains ($p > 0.05$). The lower the IC_{50} value, the greater the antioxidant activity produced by endophytic fungi. Thus, the MeOH extracts from each fungal strain displayed greater scavenging activity among the tested extracts. The total reduction power from FRAP assay was notable for all tested extracts among the fungal strains with its limit of detection at 1.6 mg/mL. The higher the FRAP reducing power, the more potent the antioxidant activity produced by endophytic fungi. The FRAP reducing power displayed in the extract of *Xylaria* sp. H4 ranged between 252.9 ± 54.8 µM (hexane) and 686 ± 35.1 µM (EtOAc), while in the extract of *Pyrrhoderma* sp. H7, the range was between 111 ± 69.2 µM (hexane) and 410.5 ± 39.5 µM (MeOH). Among the strains, the FRAP reducing power was significantly different only in the EtOAc extracts ($t = 13.05$, $p = 0.001$) and not in the MeOH and hexane extracts. Based on the antioxidant capacity and the statistical results, the MeOH and EtOAc extracts from each strain stood out as the most prominent metabolites and were selected for further analysis.

These findings showed that endophytic fungi from *Hornstedtia scyphifera* are a potential antioxidant resource worth investigating. Analysis of the possible antioxidant compounds present in the bioactive extracts was determined using an HPLC-DAD technique, by comparing the two bioactive extracts (MeOH and EtOAc) from the two fungal isolates with the reference standards (Fig. S2). The chromatograms showed that the fungal extracts had multiple peaks that to some extent matched the retention time (RT) values of the reference polyphenol antioxidants. In the current study, *Xylaria* sp. H4 and *Pyrrhoderma* sp. H7 produced five types of antioxidants in MeOH extracts: GA, catechin, chlorogenic acid, caffeic acid and luteolin. The EtOAc extract from *Pyrrhoderma* sp. H7 did not reveal any antioxidant compounds, while the EtOAc extract from *Xylaria* sp. H4 revealed only two compounds, namely CGA and caffeic acid (Table 3). These results indicated that notable antioxidant compounds were present that had been produced by potential fungal strains from *Hornstedtia scyphifera*.

Discussion

Endophytic fungi are a sustainable resource from natural products, especially phytochemicals. The antioxidant activity of fungal endophytes from medicinal plants is still being investigated due to the potential and diversity across fungal taxa and plant species as their host. The current study recovered some fungal isolates belonging to the genera *Annulohypoxylon, Ganoderma, Gliomastix, Memnoniella, Pyrrhoderma, Rostrohypoxylon, Tolypocladium* and *Xylaria*. The xylariaceous fungal taxa were dominant (represented by three taxa) as endophytic fungal species in *H. scyphifera*. The presence of fungal endophytes of the Xylariaceae have frequently been documented in other studies, for example in *Zingiber griffithii* (Zingiberaceae) (Lutfia et al., 2021) and numerous medicinal plant species in Taiwan from the Lauraceae (*Cinnamomum kanehirae, C. osmophleum, Lindera aggregata, Litsea cubeba, Machilus thunbergii*) and the Rutaceae (*Fortunella japonica*, *Zanthoxylum wutaiense*) (Ho et al., 2012).

Table 3 Detection of antioxidant compounds in fungal extracts

Compound	RT (min)	Formula	MW (g/mol)	*Xylaria* sp. H4		*Pyrrhoderma* sp. H7	
				MeOH	EtOAc	MeOH	EtOAc
Gallic acid	2.985	$C_7H_6O_5$	170.2	+	-	+	-
COG	5.221	$C_{21}H_{21}O_{11}^+$	449.4	-	-	-	-
Catechin	7.174	$C_{15}H_{14}O_6$	290.3	+	-	+	-
Chlorogenic acid	7.261	$C_{16}H_{18}O_9$	354.3	+	+	+	-
Caffeic acid	8.737	$C_9H_8O_4$	180.2	+	+	+	-
Rutin	10.552	$C_{27}H_{30}O_{16}$	610.5	-	-	-	-
Ferulic acid	11.567	$C_{10}H_{10}O_4$	194.2	-	-	-	-
Phloridzin	12.749	$C_{21}H_{24}O_{10}$	436.1	-	-	-	-
Icariin	13.225	$C_{33}H_{40}O_{15}$	676.7	-	-	-	-
Rosmarinic acid	15.089	$C_{18}H_{16}O_8$	360.3	-	-	-	-
Luteolin	15.672	$C_{15}H_{10}O_6$	286.2	+	-	+	-
Apigenin	17.562	$C_{15}H_{10}O_5$	270.1	-	-	-	-

RT = retention time; MW = molecular weight; MeOH = methanol; EtOAc = ethyl acetate; COG = cyanidin-3-O-glucoside; + = presence; - = absence

The principal antioxidant activity discovered in the current study was higher in the MeOH and EtOAc extracts, indicating the presence of abundant water-soluble antioxidants produced by endophytic fungi during fermentation. Many researchers have reported that metabolite extraction from endophytic fungus using EtOAc yielded higher amounts of antioxidants due to their high affinity to bioactive compounds (Yadav et al., 2014; Khalil et al., 2020; Silva et al., 2020;). The results could vary depending on the growth condition and extraction technique. A study by Qian et al. (2014) found that MeOH solvent extraction produced the maximum yield of phenolic compounds from fungal extracts compared to other solvents, such as acetone, ethyl acetate, chloroform and hexane. Synthesis of phenolic compounds and flavonoids, as an integrated antioxidant system by living organisms, is a means to stabilize lipid peroxidation in the cellular membrane due to extrinsic stress triggered by reactive oxygen species (ROS) (Su et al., 2019). The duration of batch fermentation in this study was 4 wk, as it was assumed that the batches would be depleted in nutrients by then, which may trigger cellular starvation and the accumulation of ROS, leading to the secretion of antioxidant compounds by the fungal isolates to thrive in the culture environment (Bai et al., 2003).

The discovery of novel antioxidant-producing agents or new microbial strains is promising for industrial or mass production due to their efficacy and safe use in chemo-preventive therapy. The five types of antioxidant compounds produced by the fungal strains in the current study are widely known as powerful key antioxidants either accumulated in medicinal plants or being produced by endophytic fungi through *in vitro* experimentation. For example, GA is a phenolic byproduct obtained during tannin hydrolysis through tannase activity that catalyzes the breakdown of the ester bonds of the compound (Weetal, 1985). Microbial production of GA using filamentous fungi has been reported with prominent members from *Aspergilli, Fusaria, Penicilii* and *Trichoderma,* mainly for industrial outputs (Bajpai and Patil, 1996). A study by Bose and Gowrie (2017) reported the detection of GA through HPLC analysis in the EtOAc extract from the root of the endophytic *Aspergillus* sp., in *Casuarina junghuhniana* Miq. In addition, GA has been used as a gold standard or unit in quantifying the antioxidant capacity of a compound produced by microorganisms.

Catechin is a polyphenolic compound with beneficial health value, due to its anticancer, antidiabetic, hepatoprotective and neuroprotective properties (Isemura, 2019). Fungal production of catechin is not usual; some studies have reported detection of the product in fungal extracts using chromatographic analysis. The endophytic fungus *Xylaria venosula* strain CBL12 from the medicinal plant *Conyza blinii* was reported to produce catechin in the EtOAc extract and it also showed good antibacterial and antioxidant properties (Tang et al., 2020). A collection of endophytic *Fusarium* spp. from *Fritillaria unibracteata* var. *wabuensis* was reported to produce catechin in five different types of extracts, following other detected compounds, such as GA, ferulic acid and rutinum in large quantities, as indicated by the highest peak based on HPLC analysis (Pan et al., 2017).

Chlorogenic acid (CGA) is an ester of caffeic acid or a phenolic compound, which possesses beneficial pharmacological value, such as anticancer, antimicrobial, antioxidant and anti-obesity properties (Naveed et al., 2018). Caffeic acid is a polyphenol in the hydroxycinnamate and phenylpropanoid metabolites, with a wide distribution in plant tissues and having bioactive values, such as antioxidant, antimicrobial and inhibitor to cyclooxygenase II (COX-2) with cytotoxicity properties (Magnani et al., 2014). Bioprospecting of either CGA or caffeic acid produced by endophytic fungi from medicinal plant is still limited, especially those derived from the Zingiberaceae. A Chinese traditional medicinal plant, *Eucommia ulmoides* Oliver, is reported to harbor a diverse chlorogenic-producing fungal species. An endophytic fungus, *Sordariomycete* sp. B5, isolated from a study, produced CGA in the ethanolic (EtOH) extract (Chen et al., 2010). An endophytic fungus, *Cladosporium velox* of *Tinospora cordifolia,* was reported as a prominent strain that produced caffeic acid with high antioxidant activity and genoprotective properties (Singh et al., 2016). A study by Das et al. (2018) detected caffeic acid from an EtOAc extract of *Fusarium chlamydosporum* and *Penicillium canescens* from *Polygonum chinense.*

Luteolin is a flavonoid widely distributed in different plant sources such as fruits, herbs, and vegetables (Aziz et al., 2018). The compound is known to possess anti-inflammatory and anticancer properties and it acts by causing intracellular ROS-triggered cell death in cancer cells (Imran et al., 2019). Despite its substantial anti-cancer properties, the microbial production of luteolin is still limited and has been explored only rarely from endophytic sources. However, Marin et al. (2017) attempted to produce this compound through *de novo* biosynthesis using *Streptomyces albus*; however, only a low amount of luteolin was produced in the laboratory.

Since flavonoids are limited in plant sources, the current study suggested the use of fungal agents as alternative cell factories to obtain the natural compounds through a fermentation technique. However, the quantity of antioxidant compounds produced by the fungal strains in the current study

is still questionable and they require further research and validation. Even though multiple antioxidant compounds were detected and could be produced by fungal strains, there is a lack of technical treatments either for the analytical technique or for the growth conditions favored by the fungal strains. A study regarding the optimization of the growth medium and the cultivating conditions should produce better results with the fungal strains. In addition, the antioxidant compounds produced by *Xylaria* and *Pyrrhoderma* strains may be seen as a bottom-up evaluation for future studies aiming to explore the bioactive capacity of *H. scyphifera* metabolites, especially as antimicrobial, antioxidant and possibly anticancer agents.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Abdelwahab, M.F., Kurtán, T., Mándi, A., et al. 2018. Induced secondary metabolites from the endophytic fungus *Aspergillus versicolor* through bacterial co-culture and OSMAC approaches. Tetrahedron Lett. 59: 2647–2652. doi.org/10.1016/j.tetlet.2018.05.067

Avanti, C., Remanti, E., Yuniarta, T.A., Azminah, A., Yunita, O., Setiawan, F. 2021. Antioxidant activity of different parts of *Nauclea subdita*. Trop. J. Nat. Prod. Res. 5: 1365–1370. doi.org/10.26538/tjnpr/v5i8.7

Aziz, N., Kim, M.Y., Cho, J.Y. 2018. Anti-inflammatory effects of luteolin: A review of *in vitro*, *in vivo*, and *in silico* studies. J. Ethnopharmacol. 225: 342–358. doi.org/10.1016/j.jep.2018.05.019

Bai, Z., Harvey, L.M., McNeil, B. 2003. Oxidative stress in submerged cultures of fungi. Crit. Rev. Biotechnol. 23: 267–302. doi.org/10.1080/07388550390449294

Bajpai, B., Patil, S. 1996. Tannin acyl hydrolase (EC 3.1. 1.20) activity of *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma*. World J. Microbiol. Biotechnol. 12: 217–220. doi.org/10.1007/BF00360918

Bose, P., Gowrie, U. 2017. Assessment of bioactive metabolites from the root endophyte isolated from *Casuarina junghuhniana* Miq. Asian J. Pharm. Clin. Res. 10: 137–143. doi.org/10.22159/ajpcr.2017.v10i4.16382

Chakraborty, A., Kundu, S., Mukherjee, S., Ghosh, B. 2019. Endophytism in Zingiberaceae: Elucidation of beneficial impact endophytes and secondary metabolites In: Jha, S. (Ed.). Endophytes and Secondary Metabolites. Springer Cham. Birkhäuser Verlag AG, Switzerland, pp. 1–26. doi.org/10.1007/978-3-319-90484-9_31

Chan, E.W.C., Wong, S.K. 2015. Phytochemistry and pharmacology of ornamental gingers, *Hedychium coronarium* and *Alpinia purpurata*: A review. J. Integr. Med. 13: 368–379. doi.org/10.1016/S2095-4964(15)60208-4

Chen, X., Sang, X., Li, S., Zhang, S., Bai, L. 2010. Studies on a chlorogenic acid-producing endophytic fungi isolated from *Eucommia ulmoides* Oliver. J. Ind. Microbiol. Biotechnol. 37: 447–454. doi.org/10.1007/s10295-010-0690-0

Das, M., Prakash, H.S., Nalini, M.S. 2018. Bioactive sesquiterpene, plasticizer, and phenols from the fungal endophytes of *Polygonum chinense* L. Annals Microbiol. 68: 595–609. doi.org/10.1007/s13213-018-1367-6

Deshmukh, S.K., Verekar, S.A., Bhave, S.V. 2015. Endophytic fungi: A reservoir of antibacterials. Front. Microbiol. 5: 715. doi.org/10.3389/fmicb.2014.00715

Fernando, O., Nursyahra, R. 2008. Utilization of Zingiberaceae as medidinal plants in Luhak Nan Duo District Pasaman Barat Regency. Pendidik. Biol. 4: 1–5. [in Indonesian]

Ginting, R.C.B., Sukarno, N., Widyastuti, U., Darusman, L.K., Kanaya, S.K. 2013. Diversity of endophytic fungi from red ginger (*Zingiber officinale* Rosc.) plant and their inhibitory effect to *Fusarium oxysporum* plant pathogenic fungi. Hayati J. Biosci. 20: 127–137. doi.org/10.4308/hjb.20.3.127

Habsah, M., Amran, M., Mackeen, M.M., et al. 2000. Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. J. Ethnopharmacol. 72: 403–410. doi.org/10.1016/s0378-8741(00)00223-3

Hartanto, S., Fitmawati, Sofiyanti, N. 2014. Ethnobotanical study of Zingiberaceae by traditional people in Pangean District Kuantan Singingi Regency, Riau. Biosaintifika 6: 98–108. [in Indonesian]

Ho, J.C. 2011. Antimicrobial, mosquito larvicidal and antioxidant properties of the leaf and rhizome of *Hedychium coronarium*. J. Chinese Chem. Soc. 58: 563–567. doi.org/10.1002/JCCS.201190021

Ho, M.Y., Chung, W.C., Huang, H.C., Chung, W.H., Chung W.H. 2012. Identification of endophytic fungi of medicinal herbs of Lauraceae and Rutaceae with antimicrobial property. Taiwania 57: 229–241. doi.org/10.6165/tai.2012.57(3).229

Imran, M., Rauf, A., Abu-Iznied, T., et al. 2019. Luteolin, a flavonoid, as an anticancer agent: A review. Biomed. Pharmacother. 112: 108612. doi.org/10.1016/j.biopha.2019.108612

Isemura, M. 2019. Catechin in human health and disease. Molecules 24: 528. doi.org/10.3390/molecules24030528

Juwita, T., Puspitasari, I.M., Levita, J. 2018. Torch ginger (*Etlingera elatior*): A review on its botanical aspects, phytoconstituents and pharmacological activities. Pakistan J. Biol. Sci. 21: 151–165. doi.org/10.3923/pjbs.2018.151.165

Kaul, S., Gupta, S., Ahmed, M., Dhar, M.K. 2012. Endophytic fungi from medicinal plants: A treasure hunt for bioactive metabolites. Phytochem. Rev. 11: 487–505. doi.org/10.1007/s11101-012-9260-6

Khalil, D.M.A., El-Zayat, S.A., El-Sayed, M.A. 2020. Phytochemical screening and antioxidant potential of endophytic fungi isolated from *Hibiscus sabdariffa*. J. Appl. Biotechnol. Rep. 7: 116–124. doi.org/10.30491/jabr.2020.109287

Kumar, S., Kaushik, N. 2013. Batch culture fermentation of endophytic fungi and extraction of their metabolites. Bio-Protocol. 3: e926. doi: 10.21769/BioProtoc.926

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35: 1547–1549. doi.org/10.1093/molbev/msy096

Lutfia, A., Munir, E., Yurnaliza. 2019a. Antagonistic endophytic fungi of *Globba pendula* Roxb. from Taman Hutan Raya, North Sumatra against *Staphylococcus aureus* ATCC® 29213™. IOP Conf. Ser. Earth Environ. Sci. 305: 012003. doi.org/10.1088/1755-1315/305/1/012003

Lutfia, A., Munir, E., Yurnaliza. 2019b. Antagonistic endophytic fungi of *Hedychium coronarium* Roxb. from Hutan Sibayak and Taman Hutan Raya, North Sumatra against *Staphylococcus aureus* ATCC® 29213™. IOP Conf. Ser. Earth Environ. Sci. 305: 012002. doi.org/10.1088/1755-1315/305/1/012002

Lutfia, A., Munir, E., Yurnaliza, Y., Hartanto, A. 2019c. Antagonistic fungal endophytes colonizing rhizome of *Amomum centrocephalum* A.D. Poulsen from North Sumatera, Indonesia. Int. J. Adv. Sci. Eng. Inf. Technol. 9: 1427–1433. doi.org/10.18517/ijaseit.9.4.8781

Lutfia, A., Munir, E., Yurnaliza, Y. 2020. Molecular identification of endophytic fungi from torch ginger (*Etlingera elatior*) antagonist to phytopathogenic fungi. *Biodiversitas* 21: 2681–2689. doi.org/10.13057/biodiv/d210641

Lutfia, A., Munir, E., Yurnaliza, Y., Basyuni, M. 2021. Chemical analysis and anticancer activity of sesterterpenoid from an endophytic fungus *Hypomontagnella monticulosa* Zg15SU and its host *Zingiber griffithii* Baker. Heliyon 7: e062292. doi.org/10.1016/j.heliyon.2021.e06292

Magnani, C., Isaac, V.L.B., Correa, M.A., Salgado, H.R.N. 2014. Caffeic acid: A review of its potential use in medications and cosmetics. Anal. Methods. 6: 3203–3210. doi.org/10.1039/C3AY41807C

Makris, D.P., Psarra, E., Kallithraka, S., Kefalas, P. 2003. The effect of polyphenolic composition as related to antioxidant capacity in white wines. Food Res. Int. 36: 805–814. doi.org/10.1016/S0963-9969(03)00075-9

Marin, L., Gutierrez-del-Rio, I., Yague, P., Manteca, A., Villar, C.J., Lombo, F. 2017. *De novo* biosynthesis of apigenin, luteolin, and eriodictyol in the actinomycete *Streptomyces albus* and production improvement by feeding and spore conditioning. Front Microbiol. 8: 921. doi.org/10.3389/fmicb.2017.00921

Munir, E., Lutfia, A., Yurnaliza, Y. 2020. Colonization of *Trichoderma* spp. in rhizome of five Zingiberaceous species from North Sumatra, Indonesia. Int. J. Agr. Biol. 24: 831–837. doi.org/10.17957/IJAB/15.1506

Naveed, M., Hejazi, V., Abbas, M., et al. 2018. Chlorogenic acid (CGA): A pharmacological review and call for further research. Biomed. Pharmacother. 97: 67–74. doi.org/10.1016/j.biopha.2017.10.064

Nongalleima, K., Dey, A., Deb, L., Singh, C.B., Thongam, B., Sunitibala, H.D., Devi, S.I. 2013. Endophytic fungus isolated from *Zingiber zerumbet* (L.) Sm. inhibits free radicals and cyclooxygenase activity. Int. J. Pharm Tech Res. 5: 301–307.

Pan, F., Su, T.J., Cai, S.M., Wu, W. 2017. Fungal endophyte-derived *Fritillaria unibracteata var. wabuensis*: Diversity, antioxidant capacities in vitro and relations to phenolic, flavonoid or saponin compounds. Sci. Rep. 7: 42008. doi.org/10.1038/srep42008

Pansanit, A., Pripdeevech, P. 2018. Antibacterial secondary metabolites from an endophytic fungus, *Arthrinium* sp. MFLUCC16-1053 isolated from *Zingiber cassumunar.* Mycology 9: 1–9. doi.org/10.1080/21501203.2018.1481154

Pulido, R., Bravo, L., Saura-Calixto, F. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agr. Food Chem. 48: 3396–3402. doi.org/10.1021/jf9913458

Putra, I.P., Rahayu, G., Hidayat, I. 2015. Impact of domestication on the endophytic fungal diversity associated with wild Zingiberaceae at Mount Halimun Salak National Park. Hayati J. Biosci. 22: 157–162. doi.org/10.1016/j.hjb.2015.10.005

Radić, N., Štrukelj, B. 2012. Endophytic fungi: The treasure chest of antibacterial substances. Phytomedicine 19: 1270–1284. doi.org/10.1016/j.phymed.2012.09.007

Qian, Y., Kang, J., Wang, L., Lei, B., Hyde, K.D. 2014. *In vitro* antioxidant and antitumor activities of an endophytic fungus *Phomopsis liquidambari* QH4 from *Artemisia annua*. Chaing Mai J. Sci. 41: 992–1006.

Shan, B., Cai, Y.Z., Brooks, J.D., Corke, H. 2007. The *in vitro* antibacterial activity of dietary spice and medicinal herb extracts. Int. J. Food Microbiol. 117: 112–119. doi.org/10.1016/j.ijfoodmicro.2007.03.003

Sholikhah, E.N. 2016. Indonesian medicinal plants as sources of secondary metabolites for pharmaceutical industry. J. Med. Sci. 48: 226–239. doi.org/10.19106/JMedSci004804201606

Silva, M.H.R., Cueva-Yesquen, L.G., Junior, S.B., Garcia, V.L., Sartoratto, A., de Angelis, D.F., de Angelis, D.A. 2020. Endophytic fungi from *Passiflora incarnata*: An antioxidant compound source. Archv Microbiol. 202: 2779–2789. doi.org/10.1007/s00203-020-02001-y

Singh, B., Sharma, P., Kumar, A., Chadha, P., Kaur, R., Kaur, A. 2016. Antioxidant and *in vivo* genoprotective effects of phenolic compounds identified from an endophytic *Cladosporium velox* and their relationship with its host plant *Tinospora cordifolia*. J. Ethnopharmacol. 194: 450–456. doi.org/10.1016/j.jep.2016.10.018

Strobel, G. 2018. The emergence of endophytic microbes and their biological promise. J. Fungi 4: 57. doi.org/10.3390/jof4020057

Su, L.J., Zhang, J.H., Gomez, H., Murugan, R., Hong, X., Xu, D., Jiang, F., Peng, Z.Y. 2019. Reactive oxygen species-induced lipid peroxidation in apoptosis, autophagy, and ferroptosis. Oxid. Med. Cell Longev. 2019: 5080843. doi.org/10.1155/2019/5080843

Tang, Z., Wang, Y., Yang, J., et al. 2020. Isolation and identification of flavonoid-producing endophytic fungi from medicinal plant *Conyza blinii* H. Lev that exhibit higher antioxidant and antibacterial activities. PeerJ 8: e8978. doi.org/10.7717/peerj.8978

Uzma, F., Konappa, N.M., Chowdappa, S. 2016. Diversity and extracellular enzyme activities of fungal endophytes isolated from medicinal plants of Western Ghats, Karnataka. Egypt. J. Basic Appl. Sci. 3: 335–342. doi.org/10.1016/j.ejbas.2016.08.007

Weetal, H.H. 1985. Enzymatic gallic acid esterification. Biotechnol. Bioeng. 27: 124–127. doi.org/10.1002/bit.260270203

Yadav, M., Yadav, A., Yadav, J.P. 2014. *In vitro* antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lan. Asian Pac. J. Trop. Med. 7: S256–S261. doi.org/10.1016/S1995-7645(14)60242-X