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Bioactivity Screening of Endophytic Fungus *Eutypa linearis* Isolated from *Coleus amboinicus* (Lour.)

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ABSTRACT

Coleus amboinicus (Lour.) is a medicinal plant containing various bioactive compounds. Endophytes are microorganisms living inside the intracellular tissue of plants and are known as the source of bioactive compounds. In order to explore the potential of endophytes in producing novel bioactive compounds, this study focused on isolating endophytic fungi from the leaves of *C.amboinicus*, characterizing, and screening their metabolite bioactivity during submerged culture fermentation. Isolation of endophytic fungi from the leaves segment of *C.amboinicus* was conducted in PDA media and fungus identification was carried out by analyzing its morphology and through molecular examination. The production of metabolites was examined using thin-layer chromatography and gas chromatography. Identification of the endophytic fungus showed 98.84% similarity with *Eutypa linearis*. This species was fermented by submersion in PDB medium for 14 days under dark and exposed to light conditions. The fermentation broth was extracted using ethyl acetate. The results showed that exposure to light influenced metabolite production. The ethyl acetate extract exhibited antioxidant and cytotoxic activities. Antioxidant activity of this extract as examined by DPPH assay showed IC_{50} of 105.31±2.11µg/mL. Cytotoxic activity against the Hela cell line was known to be the best compared with other cell lines (IC_{50} 302.28±10.30 µg/mL) although it was found to be nonselective (SI<3). The extract contained five major compounds namely benzenemethanol, 4-nitro-(CAS) p-nitrobenzyl alcohol; 2-pentadecanone (CAS) pentadecan-2-one; (1R*,6S*,10R*)-5,5-dimethyl-11,12-dioxatricyclo[8.2.1.0(1,6)] tridecan-10-ol; 9,12-cctadecadienoic acid (Z,Z)-, methyl ester (CAS) methyl linoleate; and 3-furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo- (CAS) 2-carboxymethyl-3-N-hexyl-maleic anhydride.

Keywords: *Eutypa linearis*, submerged fermentation, cytotoxicity, antioxidant, endophyte.

INTRODUCTION

Coleus amboinicus (Lour.) is one of medicinal plants that has many benefits. This plant belongs to the Lamiaceae family and is known as an aromatic herb that was used traditionally as medicine, food mixture, seasoning, and even home plant. As an herbal medicine, it was used to treat various diseases such as headaches, fever, common cold, cough, asthma, constipation, and some skin disease (Arumugam *et al*., 2016). These were suggested to correlate with the presence of volatile oil within the leaves that contain various bioactive compounds. Some of the compounds including monoterpenes, diterpenes, triterpenes, sesquiterpenes, phenolics, flavonoids, and esters. Among other parts, the leaves of *C.amboinicus* were known to be the most commonly used part. The bioactive compounds reported within the leaf essential oil were thymol, carvacrol, 1,8-cineole, *p*-cymene, spathulenol, terpinen-4-ol, β-caryophyllene, α-humulene, γ-

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terpinene, α-terpineol, eugenol, and β-seline (Arumugam *et al*., 2016; Singh *et al*., 2002). Those chemical constituents and the yields within the plants vary from one to the other and were influenced by geographical specificity and the extraction methods (Arumugam *et al*., 2016).

Since first described by Heinrich Friedrich in 1809 (Hardoim *et al*., 2015), endophyte was found to be challenging as new research object and endophyte exploration has been increasing worldwide. Endophytes that live and colonize in intracellular tissue of healthy plant (Astuti *et al*., 2014; Chen *et al*., 2016; Porras-Alfaro *and* Bayman, 2011) were known to produce secondary metabolites. *Penicillium* sp., an endophytic fungi, was reported to produce penicillenols that are cytotoxic to numerous cell lines (Lin *et al*., 2008). Other endophytic fungi could produce some compounds with antibacterial and antifungal activity such as clavatol by *Toreya mairei*, javanicin by *Chloridium* sp, jesterone by *Pestalotiopsis jester*, and sordaricin from *Fusarium* sp.(Jalgaonwala *et al*., 2011). As an antioxidant, pestacin isolated from *P.microspora* has excellent properties (Gouda *et al*., 2016). The most successful finding from the endophytic fungi study was the discovery of *Aspergillus fumigatus* producing taxol isolated from *Taxus* sp. as the alternative source due to its high yield that is needed in industrial production platforms (Kumar *et al*., 2019). In Indonesia, our group (Astuti *et al*., 2014) also discovered that endophytic fungus both from the leaves and stem of *C. amboinicus* had antimicrobial activity against some bacteria. We discovered another strain of endophytic fungus which is potential to be screened for its bioactive metabolites. Therefore, this study focused on the screening of phytochemical contents as well as bioactivity from metabolites produced by submerged culture fermentation of the newly isolated endophyte.

MATERIAL AND METHODS

Plant materials and endophytic fungi isolation

C.amboinicus was obtained from Pharma Green House, Faculty of Pharmacy, Universitas Gadjah Mada in September 2019 and authenticated by Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada. The voucher specimen was collected under reference number 014745. Endophytic fungi were isolated from the third leaves counted from the shoots of *C.amboinicus* aseptically according to Astuti *et al* (2014) with slight modification. Briefly, the l eaves were washed with running tap water and detergent for 5min. Under laminar airflow hood, the leaves were dipped in 70% ethanol for 5min to wet the surface and surface-sterilized for 5min in a solution of 1% NaOCl. Leaves were then rinsed three times in sterile distilled water for 1, 3, and 5min and dried on sterile filter paper. Segments were obtained by excising the surface-sterilized leaves into 1cm^2 aseptically with a sterile knife blade. The open-inner tissue was then placed onto potato dextrose agar (PDA) medium mixed with 50μg/mL chloramphenicol in a sterile petri dish. Dishes then were incubated at 25°C until the hyphal tip of endophytic fungi grew pointing out the segment resulting in a primary source of endophytic fungi. In order to obtain the pure culture of isolated fungi, the inoculum from the primary source of endophytic fungi was subcultured onto other PDA plates.

Identification of endophytic fungi

The pure culture of fungus was identified by the Indonesian Culture Collection Lembaga Ilmu Pengetahuan Indonesia (LIPI). The morphological examination was conducted by phenotype identification macroscopically and microscopically according to Hawksworth (1974), Webster (1980), Domsch et.al. (1980), Ellis (1971), Samson *et al* (1995), Alexopoulos (2007), and Barnett *and* Hunter (1998). Light microscope Olympus BX53 and Olympus DP26 camera was used in specimen observation to obtain microscopic visualization. Molecular identification of the pure endophytic fungus was conducted as partial genetic analysis for ribosomal DNA (rDNA) at the internal transcribed spacer (ITS) region. DNA obtained was amplified using a pair of primer; 5'-TCC TCC GCT TAT TGA TAT GC-3' as ITS 4 primer region and 5`–GGA AGT AAA AGT CGT AAC AAG G –3` as ITS 5 primer region. The amplicon was sequenced and confirmed using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and BLAST analysis on DNA Data Bank of Japan website (http://blast.ddbj.nig.ac.jp/) or National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/) to determine the species with the highest molecular similarity.

Endophytic fungi fermentation

The isolated strain of fungus was grown in PDA medium at 25°C for 14 days. Fermentation was conducted in a 250mL Erlenmeyer flask containing 200mL potato dextrose broth medium with

initial pH of 5.0. The medium was sterilized by autoclave at 12°C for 20min prior to use and cooled to room temperature. The fermentation medium was inoculated with 4 plugs of isolated fungus using a 5mm diameter cork hole and the culture was further incubated at room temperature with 120rpm shaking for 14 days (Astuti *et al*., 2014).

Effect of light and age of inoculum to phytochemical profile

The endophytic fungus was inoculated on PDA and incubated for 7, 14, and 21 days. Four plugs of each culture were transferred into a 200mL PDB medium and fermented under a submerged condition in the presence and absence of light. Following incubation, the supernatants were harvested and extracted by liquid-liquid extraction with ethyl acetate in a ratio of 1:2 (supernatant:solvent) to gain a liquid extract. The liquid extract was further concentrated using a rotary evaporator (Heidolph Hei-Vap) at 40°C temperature and 160 mbar pressure, and subjected to phytochemical profiling.

Qualitative shytochemical screening

The profile of total metabolite produced by the endophytic fungus during the fermentation process was confirmed using thin-layer chromatography (TLC) and fingerprint analysis. The dry extract was dissolved in ethyl acetate, spotted on TLC Silica gel 60 F_{254} (Merck) plate, and eluted using chloroform:ethyl acetate (1:1) as the mobile phase. The obtained spots were visualized with a UV lamp and the phytochemical groups were determined using dyeing reagents such as anisaldehyde-sulfuric acid, vanillin-sulfuric acid, Dragendorff, $AlCl_3$, and $FeCl_3$. The fingerprint was observed using high-performance thin-layer chromatography (HPTLC). Using the same plate and mobile phase, as much as 5 µL of 100 mg/mL ethyl acetate-diluted extract was applied using Linomat V applicator. Samples were eluted and air-dried before scanned at 254 nm and 366 nm using a Deuterium light source in the CAMAG HPTLC system. Slit dimensions were set at 4.00x0.3mm. The presence of volatile compounds was determined using the GCMS-QP2010 system (Shimadzu, Tokyo, Japan). As much as 0.5µL extract diluted in chloroform:methanol (1:1) were injected using an autosampler and run through capillary column cross bond 100% dimethyl polysiloxane 30m x 0.25mm x 0.25µm film thickness Rxi-1ms.

The flow rate of helium as the carrier gas was set at 1.33 mL/min at 100 kPa. The oven and injection temperatures were maintained at 100°C and 250°C with a sampling time of 1min. The split ratio was 1:50. The mass detector electron ionization was 70eV. Identification of volatile compounds was carried out using mass spectra WILEY7.LIB library search.

Bioactivity screening

Antioxidant activity

Antioxidant activity was determined using the scavenging effect of DPPH radicals (Sigma-Aldrich Co., St. Louis, MO, USA). Five different concentrations of extract were made in methanol, each of which was mixed with 0.1mM DPPH solution in methanol. The solutions were left in dark to complete the reaction for 30 minutes. Absorbance at 517nm (Spectrophotometer UV-Vis) was used to calculate the radical scavenging activity as percent of inhibition described by de Torre *et al* (2019) using the following formula:

$$Inhibition\ (\%) = 1 - \frac{Abs\ Sample - Abs\ blank}{Abs\ control - Abs\ blank} \times 100$$

where Abs sample is the absorbance of each sample solution reacted with DPPH, Abs blank is the absorbance of the blank for each sample dilution and Abs control is the absorbance of the DPPH solution. The values obtained were plotted to determine IC_{50} of the extract as the concentration that gives a 50% reduction in the activity of the DPPH free radicals.

Cytotoxic assay

Cytotoxic activity on WiDr, MCF7, Hela, T47D, and Vero cell lines was assessed by MTT (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide) assay by the method of Mosmann (1983) with slight modification. The cell lines were obtained from the Medical Parasitology Laboratory, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada. A complete growth medium was provided for cell growth: Dulbecco's modified Eagle medium (DMEM) for MCF7 and Vero, and RPMI medium for WiDr, Hela, and T47D, each supplemented with 10% v/v Fetal Bovine Serum, 2% v/v Penicillin-Streptomycin, and 0.5% v/v Amphotericin B. A total of 10^4 cells/well were plated in 96 well-culture plates and permitted to attach for a period of 24h, with the temperature maintained

at 37°C and 5% CO_2 in a humidified incubator. The medium was then replaced, and cells were exposed to 100µL of different concentrations of extract sample dissolved in DMSO or doxorubicin as a control treatment and further incubated for 24h in an incubator. The medium was then aspirated, MTT (5mg/mL of stock in PBS) was added (10µL/well in 100µL of cell suspension), and cells were incubated for additional 4 hours. At the end of the incubation period, 100µL of SDS (10% in HCl 0.01N) was added to each well and incubated for 24h. The absorbance of the solution in each well was measured in a microplate reader at 595nm. Results were expressed as the percent of cell viability from three independent experiments. The result was calculated as the percentage of viability using the following equation:

$$\textit{Viability}\ (\%) = \frac{\textit{Abs of test group} - \textit{Abs medium}}{\textit{Abs cell} - \textit{Abs medium}} \times 100$$

The concentration of extract needed to inhibit cell growth by 50% (IC_{50}) was generated from the dose-responsive curve for each cell line. The selectivity index (SI) of the extract was determined by calculating the ratio of IC_{50} value obtained in Vero to those of other cancer cell lines tested. The greater SI values indicate the safer compound with values of more than 3 indicating high selectivity (Prayong *et al.*, 2008; Wiji Prasetyaningrum *et al.*, 2018)

Data analysis

Each data was expressed as mean ± SD. Statistical significance was evaluated using the One-Way ANOVA test or Kruskal Wallis test (SPSS program version 16) depending on the normality value. P-values less than 0.05 were considered to be significant with a 95% confidence interval.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

The fungal strain was isolated from the fresh leaves of *C. amboinicus*. A white hyphal tip of fungi growing out from the edge of the segment in the PDA plate was further incubated on a PDA plate and appeared as a cottony and filamentous form of colony in flat elevation and has a filiform marginal. Hyaline structure (3-5µm) was found during microscopic observation (Figure 1). Analysis using ITS rDNA sequence on a BLAST search showed that the isolated fungus had similarity to existing sequences of *E. linearis* with 98.84% homology (Supplementary data 1).

Figure 1. a. Endophytic fungus isolated from leaves of *C.amboinicus* on PDA plate at 5 days of incubation. b. Hyaline form on microscopic observation.

Effect of light exposure and age of inoculum on phytochemical profiling

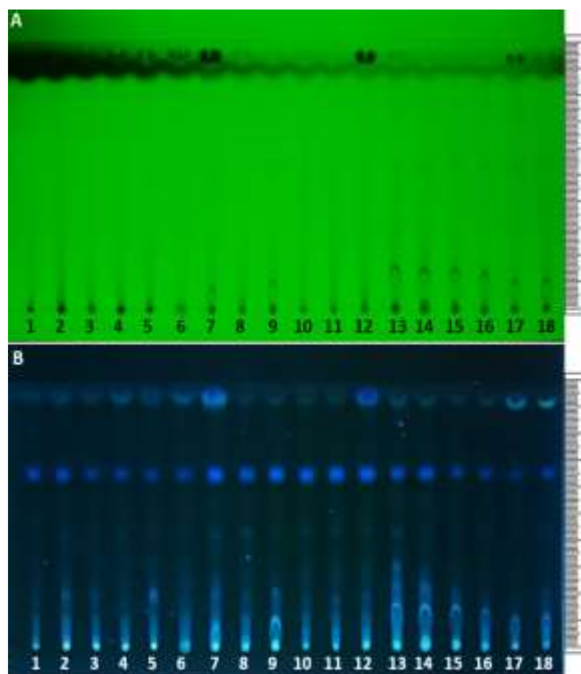

Figure 2. Effect of light during fermentation on TLC profile of ethyl acetate extract from broth medium of *E.linearis*. **A.** Observed under UV_{254} light. **B.** Under UV_{366} light. All were performed in 3 replicates and plotted on a silica gel 60 F_{254} plate with chloroform:ethyl acetate (1:1) mobile phase. 1-3: 7 days old culture incubated under light; 4-6: 7 days old culture incubated in dark condition; 7-9: 14 days old culture incubated under light; 10-12: 14 days old culture incubated in dark condition; 13-15: 21 days old culture incubated under light; 16-18: 21 days old culture incubated in dark condition. The cultures were further fermented in PDB medium for 14 days.

In general, the age of inoculum and light exposure affected the TLC profile of metabolite produced, although the difference was not major (Figure 2). More spots appeared after two weeks of incubation, which were reduced on three weeks incubation. There were some spots (Rf = ±0.43, 0.62 under UV_{366} light) showed up in all samples tested. When observed using an HPTLC fingerprint with a quantitative concentration of extract, a distinctive finding was revealed. All samples observed showed a different profile of metabolites (Figure 3).

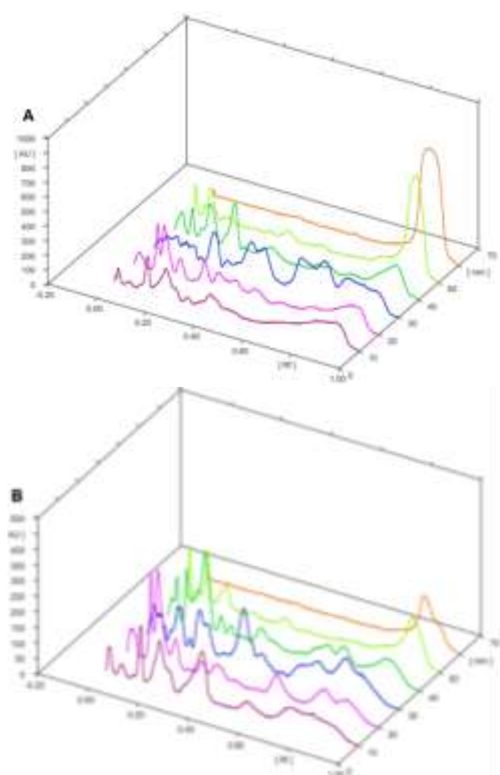

Figure 3. Dimensional fingerprint of ethyl acetate extract from broth medium of *E.linearis* scanned at 254 nm **(A)**; 366 nm **(B)**. TLC run in silica gel 60 F_{254} plate with mobile phase ethyl acetate:chloroform (1:1). Air-based drying device in 27°C temperature. 10mm: 7 days old culture incubated under light; 20mm: 7 days old culture incubated in dark condition; 30mm: 14 days old culture incubated under light; 40mm: 14 days old culture incubated in dark condition; 50mm: 21 days old culture incubated under light; 60mm: 21 days old culture incubated in dark condition. All samples were tested at 100mg/mL in ethyl acetate.

Among the samples, the most varied peaks detected were in extract fermented under light exposure using 14 days old inoculum. This study proved that the age of inoculum and light exposure during fermentation affected the metabolite produced by endophytic fungus *E. linearis*. At the same age of inoculum, light exposure could induce higher variation in chemical metabolites in *E. linearis* submerged fermentation. This premise was shown in 14- and 21-days old inoculum. Using 7 days old inoculum, light exposure did not show any significant differences in the number of metabolites produced. These variables' effect was similarly reported in a previous study. The age of fungal inoculum has effects on both mycelial growth during submerged fermentation and metabolite production. The optimum inoculum age of *Penicillium sp*., for example, was known to produce the highest amount of red pigment; an increase of inoculum age generated a decrease in mycelial growth (Gunasekaran *and* Poorniammal, 2008). In another study of pigment-producing fungus, it was found that increasing inoculum age of *Aspergillus terreus* in PDA medium resulted in a decrease in biomass and pigment production in submerged fermentation (Akilandeswari *and* Pradeep, 2017). A study on citric acid production by fungal spore of *Aspergillus niger* showed a similar result. The lower and higher than optimum inoculum age resulted in the lower production of citric acid. Those findings might be due to the maturity in fungal spores to sufficiently grow and produce citric acid (Alam *et al*., 2011).

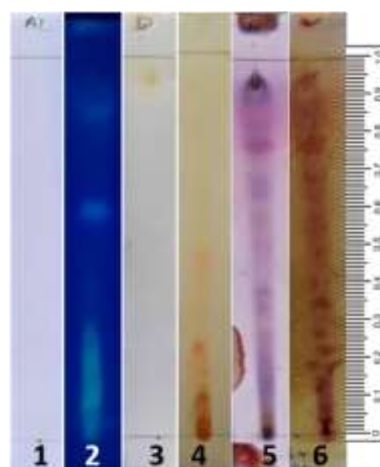

Figure 4. Phytochemical analysis of ethyl acetate extract from broth medium of *E. linearis* detected by dyeing reagents. Silica gel 60 F_{254} was used as the stationary phase with mobile phase chloroform:ethyl acetate (1:1). Dyeing reagent **1:** $AlCl_3$ visible light**; 2:** $AlCl_3$ at UV_{366}**; 3:** Dragendorff**; 4:** $FeCl_3$**; 5:** Anisaldehyde-sulfuric acid**; 6:** Vanillin sulfuric acid.

Table I. Dyeing reagent-base phytochemical screening

No.	Dyeing reagent	Response
1.	$AlCl_3$	+
2.	Dragendorff	-
3.	$FeCl_3$	+
4.	Anisaldehyde-sulfuric acid	+
5.	Vanillin-sulfuric acid	+

Phytochemical profiles

The phytochemical analysis of ethyl acetate extract from broth medium of *E. linearis* (Table I, Figure 4) exhibited the possible presence of compounds. A positive result using $AlCl_3$ dyeing reagent appeared as yellow fluorescence under long-wave UV light, indicating the presence of flavonoid (Cai *et al*., 2011; Maya *et al*., 2019). Aluminum in $AlCl_3$ reacts with flavonoids, forming aluminum-flavonoid complexes having absorbance at long-wave UV (Pękal and Pyrzynska, 2014). In this study, alkaloids detection using Dragendorff reagent did not show the appearance of yellow zones. Yellow-brownish colorization was formed due to the potassium- alkaloid complex which could be seen under visible light (Parbuntari *et al*., 2018). The absence of this colorization might imply that the alkaloid compounds were not detected either due to its absence or its inadequate yield to be detected using this method. Orange-brownish spots on TLC plate by $FeCl_3$ spraying reagent possibly indicated the presence of phenolic compounds (Pascual *et al*., 2002). The addition of $FeCl_3$ as a qualitative test reagent into samples containing phenolic compounds could produce either blue, violet, or red and orange coloration. The hydroxyl group in those compounds was known to be the group responsible for colorization (Wesp *and* Brode, 1934). Compounds such as sugars, steroids, and terpenes were detected based on the positive response to universal reagent anisaldehyde-sulfuric acid dyeing reagent and possibly alcohol, phenols, steroid, and essential oils based on response to vanillin-sulfuric acid (Jork, 1990). These findings were similar to that reported by Arumugam *et al* (2016) which showed that despite the presence of essential oils as volatile compounds in *C. amboinicus*, non-volatile constituents were also identified including phenolic acid, flavonoids, monoterpene, and sesquiterpene hydrocarbons, oxygenated monoterpenes, and esters. Similarly, the presence of sugar, terpenes, and phenolic compounds was also reported in ethanolic and aqueous extracts of the leaf of *C. amboinicus* (Patel *et al*., 2010). These findings indicated that there was some similarity of compounds produced by the leaf and endophytic fungi isolated from this part of the plant.

Additional analysis of volatile chemical compounds in the ethyl acetate extract of broth medium using gas chromatography coupled with mass spectroscopy (GC-MS) revealed the presence of five major compounds (Table II; Figure 5). The high similarity index implied highly similar compounds were detected in the sample to those suggested by the MS database. A threshold similarity index of ≥ 60 or 70% is usually set for the spectral result. The correct identification was terminated by the highest similarity index of a mass spectrum fulfilling that threshold (Kim *and* Zhang, 2015). The result revealed three major compounds with the highest similarity. They were benzenemethanol, 4-nitro-(CAS) p-nitrobenzyl alcohol, 9,12-octadecadienoic acid (Z,Z)-, methyl ester (CAS) methyl linoleate, and 2-pentadecanone (CAS) pentadecan-2-one. Some of these compounds were reported to be also found in leaf extract of *C. amboinicus.* Swamy *et al* (2017) reported 2-pentadecanone and 9,12-octadecadienoic in methanolic and acetone extracts of *C. amboinicus* leaves, respectively. Specifically, no report exists about those 3 other compounds as the major volatile compounds of *C. amboinicus* leaf extract. The alcohol which was reported by Arumugam (2016) as one of the major volatile compounds in *C. amboinicus* leaf extract, was also present in this study. TLC profile which gave a positive response to $FeCl_3$ and vanillin-sulfuric acid might correspond to the presence of p-nitrobenzyl alcohol. A group of benzyl alcohol was reported to be produced by fungi due to its reductive activity to carbonyl compounds through the fermentation process (Zhuk *et al*., 2021). These findings suggested that some compounds might be produced by endophytic fungi which were similar to those produced by the host plant.

Bioactivity

The DPPH radical scavenging activity of the extract (Table III) as compared with a known antioxidant, quercetin. The result revealed that the extract had antioxidant activity. The IC_{50} value was 105.31±2.11μg/mL, indicating a weak antioxidant activity (Phongpaichit *et al*., 2007). DPPH antioxidant activity was based on the reaction of DPPH (α, α-diphenyl-β-picrylhydrazyl) free radical with compounds that can donate hydrogen atoms through reduction reaction (Kedare and Singh, 2011).

Table II. Phytochemical components in broth medium of ethyl acetate extract of *E. linearis*

No.	RT (min)	Compound	PA (%)	SI	Molecular formula	MW (g/mol)
1.	11.115	Benzenemethanol, 4-nitro-(CAS) p-Nitrobenzyl alcohol	19.80	95%	$C_7H_7NO_3$	153
2.	12.504	2-Pentadecanone (CAS) Pentadecan-2-one	14.69	95%	$C_{15}H_{30}O$	226
3.	13.106	(1R*,6S*,10R*)-5,5-Dimethyl-11,12-dioxatricyclo[8.2.1.0(1,6)]tridecan-10-ol	9.73	76%	$C_{13}H_{22}O_3$	226
4.	14.701	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS) Methyl linoleate	15.32	94%	$C_{19}H_{34}O_2$	294
5.	14.758	3-Furanacetic acid, 4-hexyl-2,5-dihydro-2,5-dioxo- (CAS) 2-carboxymethyl-3-N-hexyl-maleic anhydride.	40.46	74%	$C_{12}H_{16}O_5$	240

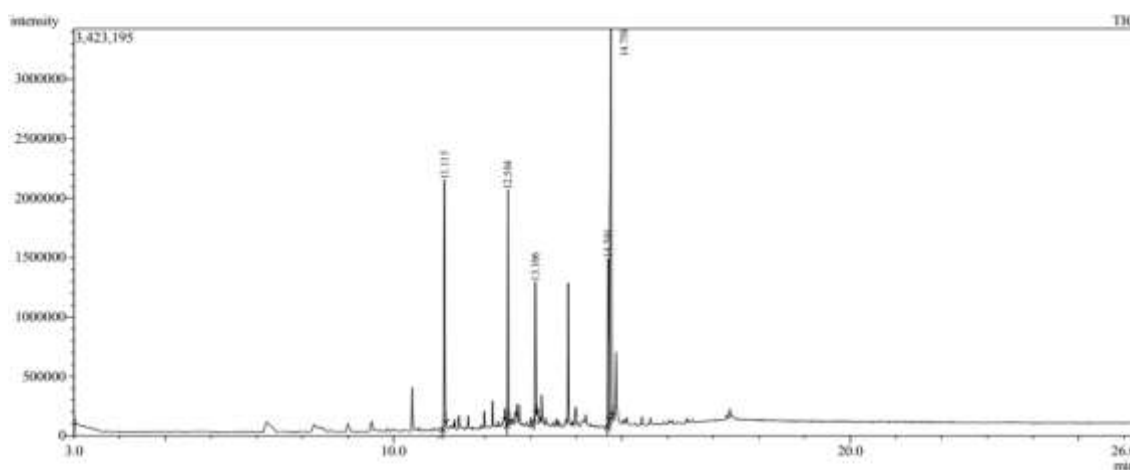

Figure 5. GC-MS profile of ethyl acetate extract of broth medium fermentation of *E. linearis*

Table III. IC_{50} value of the extract of broth medium fermentation of *E. linearis* in DPPH scavenging assay

Samples	IC_{50} (μg/mL)
Extract	105.31 ± 2.11
Quercetin	1.30 ± 0.01

All the values are expressed as mean ± SD (n=3) $P<0.05$

Table IV. IC_{50} values of the extract of broth medium fermentation of *E. linearis* and doxorubicin control against several cell lines

Cells	IC_{50} (μg/mL)		Selectivity Index (SI)	
	Extract	Doxorubicin	Extract	Doxorubicin
Hela	302.28 ± 10.30	3.56 ± 0.02	1.26 ± 0.04	2.22 ± 0.01
T47D	310.45 ± 2.58	0.56 ± 0.02	1.22± 0.01	13.98 ± 0.41
WiDr	318.13 ± 2.42	2.78 ± 0.03	1.19 ± 0.01	2.84 ± 0.03
MCF-7	714.46 ± 38.57	3.43 ± 0.08	0.53 ± 0.03	2.30 ± 0.06
Vero	379.86 ± 5.48	7.86 ± 0.87	-	-

All the values are expressed as mean ± SD (n=3) $P<0.05$

Antioxidant activity of extract most often correlates with phenolic compounds (Swamy *et al.*, 2017) which were also suggested to be produced by *E. linearis* according to a previous positive result with $FeCl_3$ dyeing reagent. This potential antioxidant activity of ethyl acetate extract of *E. linearis* was in line with previous findings which reported that leaves extract and essential oil of *C.amboinicus* also exhibited antioxidant activity by inhibiting DPPH free radicals (Bhatt *and* Negi, 2012; Kumaran *and* Joel karunakaran, 2006; Manjamalai, 2012).

To determine the bioactivity of the extract, cytotoxic activities were also tested using a series of cancer cell lines *in vitro*. Five cell lines were used during this study including MCF7, T47D, WiDR, and Hela cells were used as cancer cell models and Vero cells were used as the normal cell model. *In vitro* activity of the extract showed that the highest potential as a cytotoxic agent was against Hela cells, followed by T47D and WiDr cells (Table IV). However, the cytotoxicity of the extract was very low compared with doxorubicin. The cytotoxic activity against MCF7 even showed a higher IC_{50} value reaching 714.46 ± 38.57 μg/mL. This finding was quite different from that reported by Hasibuan *et al* (2013) who found that ethyl acetate extract of leaf of *C. amboinicus* had high cytotoxic activity on MCF-7 with an IC_{50} value of 7.467 μg/mL (Hasibuan *et al.*, 2013). The results suggested that the components within the leaves' extract and the endophytic fungus reported in this study might be different from those in the previous report.

Atjanasuppat *et al*. (2009) classified cytotoxic activity potential of extracts based on the IC_{50} value obtained. The IC_{50} value ≤ 20 μg/mL is categorized as active; > 20 – 100 μg/mL as moderately active; >100 – 1000 μg/mL as weakly active; and > 1000 μg/mL as potentially inactive. Hence, the finding of this study revealed a moderate potential activity of the extract against several models of cancer cells. The selectivity index (SI) value of the extract on each cell line investigated in this study was found lower than the SI value of Doxorubicin. It was indicated that the extract was not superior in selectivity properties compared with doxorubicin on cancer cell lines used in this study.

CONCLUSION

In this study, the endophytic fungus isolated from the leaf tissue of *C. amboinicus* was identified as *E. linearis*. The ethyl acetate extract of the fungi produced various non-volatile and volatile compounds as detected by TLC, HPTLC, and GC-MS. During fermentation, the metabolite profiles were influenced by the age of inoculum and light exposure. The extract showed weak antioxidant and cytotoxic activities.

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REFERENCES

Akilandeswari, P., *and* Pradeep, B. V. 2017. Aspergillus terreus KMBF1501 a Potential Pigment Producer under Submerged Fermentation. *International Journal of Pharmacy and Pharmaceutical Sciences*, *9*(4), 38. https://doi.org/10.22159/ijpps.2017v9i4.16176

Alam, Md. Z., Bari, Md. N., Muyibi, S. A., Jamal, P., *and* Al Mamun, A. 2011. Development of Culture Inoculum for Scale-Up Production of Citric Acid from Oil Palm Empty Fruit Bunches by *Aspergillus niger*. *Procedia Environmental Science*, *8*, 396–402.

Arumugam, G., Swamy, M. K., *and* Sinniah, U. R. 2016. *Plectranthus amboinicus* (Lour.) Spreng: Botanical, Phytochemical, Pharmacological and Nutritional Significance. *Molecules (Basel, Switzerland)*, *21*(4), 369. https://doi.org/10.3390/molecules21040369

Astuti, P., Sudarsono, S., Nisak, K., *and* Nugroho, G. W. 2014. Endophytic Fungi Isolated from *Coleus amboinicus* Lour Exhibited Antimicrobial Activity. *Advanced Pharmaceutical Bulletin; EISSN 2251-7308*. https://doi.org/10.5681/apb.2014.088

Atjanasuppat, K., Wongkham, W., Meepowpan, P., Kittakoop, P., Sobhon, P., Bartlett, A., *and* Whitfield, PJ. 2009. In vitro screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. *Journal of Ethnopharmacology*, *123*(3), 475–482. https://doi.org/10.1016/j.jep.2009.03.010

Barnett, H. L., *and* Hunter, B. B. 1998. *Illustrated Genera of Imperfect Fungi* (4th Edition). Prentice-Hall, Inc.

Bhatt, P., *and* Negi, PS. 2012. Antioxidant and Antibacterial Activities in the Leaf Extracts of Indian Borage (*Plectranthus amboinicus*).

Food and Nutrition Sciences, *3*(2), 146–152. https://doi.org/10.4236/fns.2012.32022

Cai, L.-Y., Shi, F.-X., *and* Gao, X. 2011. Preliminary phytochemical analysis of *Acanthopanan trifoliatus* (L.) Merr. *Journal of Medicinal Plants Research*, *5*(17), 4059–4064.

Chen, L., Zhang, Q.-Y., Jia, M., Ming, Q.-L., Yue, W., Rahman, K., Qin, L.-P., *and* Han, T. 2016. Endophytic fungi with antitumor activities: Their occurrence and anticancer compounds. *Critical Reviews in Microbiology*, *42*(3), 454–473. https://doi.org/10.3109/1040841X.2014.959892

de Torre, MP., Cavero, RY., Calvo, MI., *and* Vizmanos, JL. 2019. A Simple and a Reliable Method to Quantify Antioxidant Activity In Vivo. *Antioxidants*, *8*(5), 142. https://doi.org/10.3390/antiox8050142

Domsch, K. H., Gams, W., *and* Anderson, T.-H. 1980. *Compendium of Soil Fungi*. Academic Press.

Ellis, M. B. 1971. *Dematiaceous Hyphomycetes*. Commonwealth Mycological Institute.

Gouda, S., Das, G., Sen, S. K., Shin, H.-S., *and* Patra, J. K. 2016. Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01538

Gunasekaran, S., *and* Poorniammal, R. 2008. Optimization of fermentation conditions for red pigment production from *Penicillium* sp. Under submerged cultivation. *African Journal of Biotechnology*, *7*(12), 1894–1898. https://doi.org/10.5897/AJB2008.000-5037

Hardoim, P. R., Overbeek, L. S. van, Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., *and* Sessitsch, A. 2015. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiology and Molecular Biology Reviews*, *79*(3), 293–320. https://doi.org/10.1128/MMBR.00050-14

Hasibuan, PAZ., Ilyas, S., *and* Nasution, MP. 2013. Antioxidant and Cytotoxic Activities of *Plectranthus amboinicus* (Lour.) Spreng. Extracts. *International Journal of Pharmacy Teaching and Practice*, *4*(3), 755–758.

Hawksworth, D. L. 1974. *Mycologist's Handbook: An Introduction to the Principles of Taxonomy and Nomenclature in the Fungi and Lichens*. Commonwealth Mycological Institute.

Jalgaonwala, R., Mohite, B. V., *and* Mahajan, R. 2011. A review: Natural products from plant associated endophytic fungi. *J. Microbiol. Biotechnol. Res*, *1*, 21–32.

Jork, H. (Ed.). 1990. *Thin-layer chromatography: Reagents and detection methods*. VCH.

Kedare, SB., *and* Singh, RP. 2011. Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, *48*(4), 412–422. https://doi.org/10.1007/s13197-011-0251-1

Kim, S., *and* Zhang, X. 2015. Discovery of False Identification Using Similarity Difference in GC-MS based Metabolomics. *Journal of Chemometrics*, *29*(2), 80–86. https://doi.org/10.1002/cem.2665

Kumar, P., Singh, B., Thakur, V., Thakur, A., Thakur, N., Pandey, D., *and* Chand, D. 2019. Hyper-production of taxol from *Aspergillus fumigatus*, an endophytic fungus isolated from Taxus sp. Of the Northern Himalayan region. *Biotechnology Reports*, *24*. https://doi.org/10.1016/j.btre.2019.e00395

Kumaran, A., *and* Joel karunakaran, R. 2006. Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. *Food Chemistry*, *97*(1), 109–114. https://doi.org/10.1016/j.foodchem.2005.03.032

Lin, Z.-J., Lu, Z.-Y., Zhu, T.-J., Fang, Y.-C., Gu, Q.-Q., *and* Zhu, W.-M. 2008. Penicillenols from *Penicillium* sp. GQ-7, an endophytic fungus associated with Aegiceras corniculatum. *Chemical and Pharmaceutical Bulletin*, *56*(2), 217–221. https://doi.org/10.1248/cpb.56.217

M. Blackwell Constantine J. Alexopoulos, C. W. M. 2007. *Introductory Mycology, 4Th Ed* (4th edition). Wiley.

Manjamalai, A. 2012. Volatile constituents and antioxidant property of essential oil from *Plectranthus amboinicus* (Lour). *Int. J. Pharm. Biol. Sci*, *3*, 445–458.

Maya, M., Ramaniah, I., Venkatakrishna, K., Rameshkumar, K., Veeramanikandan, V., Eyini, M., *and* Balaji, P. 2019. Investigation of Bioactive Compounds of *Capsicum frutescence* and *Annona muricata* by Chromatographic Techniques. *Journal of Drug Delivery and Therapeutics*, *9*(4), 485–495. https://doi.org/10.22270/jddt.v9i4.3083

Mosmann, T. 1983. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to

Proliferation and. *Journal of Immunological Methods*, *65*, 55–63.

Parbuntari, H., Prestica, Y., Gunawan, R., Nurman, M. N., *and* Adella, F. 2018. Preliminary Phytochemical Screening (Qualitative Analysis) of Cacao Leaves (*Theobroma cacao* L.). *EKSAKTA: Berkala Ilmiah Bidang MIPA*, *19*(2), 40–45. https://doi.org/10.24036/eksakta/vol19-iss2/142

Pascual, M. E., Carretero, M. E., Slowing, K. V., *and* Villar, A. 2002. Simplified Screening by TLC of Plant Drugs. *Pharmaceutical Biology*, *40*(2), 139–143. https://doi.org/10.1076/phbi.40.2.139.5849

Patel, R., Mahobia, N., Waseem, N., Upwar, N., *and* Singh, S. 2010. Phyto-Physicochemical Investigation of Leaves of *Plectranthus amboinicus* (Lour) Spreng. *Pharmacognosy Journal*, *2*(13), 536–542. https://doi.org/10.1016/S0975-3575(10)80057-4

Pękal, A., *and* Pyrzynska, K. 2014. Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. *Food Analytical Methods*, *7*(9), 1776–1782. https://doi.org/10.1007/s12161-014-9814-x

Phongpaichit, S., Nikom, J., Rungjindamai, N., Sakayaroj, J., Hutadilok-Towatana, N., Rukachaisirikul, V., *and* Kirtikara, K. 2007. Biological activities of extracts from endophytic fungi isolated from Garcinia plants: Biological activities of extracts from endophytic fungi. *FEMS Immunology and Medical Microbiology*, *51*(3), 517–525. https://doi.org/10.1111/j.1574-695X.2007.00331.x

Porras-Alfaro, A., *and* Bayman, P. 2011. Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. *Annual Review of Phytopathology*, *49*(1), 291–315. https://doi.org/10.1146/annurev-phyto-080508-081831

Prayong, P., Barusrux, S., *and* Weerapreeyakul, N. 2008. Cytotoxic activity screening of some indigenous Thai plants. *Fitoterapia*, *79*(7–8), 598–601. https://doi.org/10.1016/j.fitote.2008.06.007

Samson, RA. 1995. *Introduction to Food-borne Fungi*. Centraalbureau voor Schimmelcultures.

Singh, G., Singh, O. P., Prasad, Y. R., Lampasona, M. P. de, *and* Catalan, C. 2002. Studies on essential oils, Part 33: Chemical and insecticidal investigations on leaf oil of *Coleus amboinicus* Lour. *Flavour and Fragrance Journal*, *17*(6), 440–442. https://doi.org/10.1002/ffj.1123

Swamy, M. K., Arumugam, G., Kaur, R., Ghasemzadeh, A., Yusoff, M. Mohd., *and* Sinniah, UR. 2017. GC-MS Based Metabolite Profiling, Antioxidant and Antimicrobial Properties of Different Solvent Extracts of Malaysian *Plectranthus amboinicus* Leaves. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2017*. https://doi.org/10.1155/2017/1517683

Webster, J. 1980. *Introduction to Fungi* (2nd edition). Cambridge University Press.

Wesp, E. F., *and* Brode, W. R. 1934. The Absorption Spectra of Ferric Compounds. I. The Ferric Chloride—Phenol Reaction. *Journal of the American Chemical Society*, *56*(5), 1037–1042. https://doi.org/10.1021/ja01320a009

Wiji Prasetyaningrum, P., Bahtiar, A., *and* Hayun, H. 2018. Synthesis and Cytotoxicity Evaluation of Novel Asymmetrical Mono-Carbonyl Analogs of Curcumin (AMACs) against Vero, HeLa, and MCF7 Cell Lines. *Scientia Pharmaceutica*, *86*(2). https://doi.org/10.3390/scipharm86020025

Zhuk, TS., Skorobohatko, OS., Albuquerque, W., *and* Zorn, H. 2021. Scope and limitations of biocatalytic carbonyl reduction with white-rot fungi. *Bioorganic Chemistry*, *108*, 104651. https://doi.org/10.1016/j.bioorg.2021.104651