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Research Article

Endophytic Fungi Isolated from *Coleus amboinicus* Lour Exhibited Antimicrobial Activity

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Abstract

Purpose: *Coleus amboinicus* is a medicinal plant traditionally used to treat various diseases such as throat infection, cough and fever, diarrhea, nasal congestion and digestive problems. The plant was explored for endophytic fungi producing antimicrobial agents.

***Methods*:** Screening for endophytic fungi producing antimicrobial agents was conducted using agar plug method and antimicrobial activity of promising ethyl acetate extracts was determined by disc diffusion assay. Thin layer chromatography (TLC) - bioautography was performed to localize the bioactive components within the extract. TLC visualization detection reagents were used to preliminary analyze phytochemical groups of the bioactive compounds.

***Results*:** Three endophytic fungi were obtained, two of them showed promising potential. Agar diffusion method showed that endophytic fungi CAL-2 exhibited antimicrobial activity against *P. aeruginosa*, *B. subtilis*, *S. aureus* and *S. thypi*, whilst CAS-1 inhibited the growth of *B. subtilis*. TLC bioautography of ethyl acetate extract of CAL-2 revealed at least three bands exhibited antimicrobial activity and at least two bands showed inhibition of *B. subtilis* growth. Preliminary analysis of the crude extracts suggests that bioactive compounds within CAL-2 extract are terpenoids, phenolics and phenyl propanoid compounds whilst the antimicrobial agents within CAS-1 extract are terpenoids, propylpropanoids, alkaloids or heterocyclic nitrogen compounds.

***Conclusion*:** These data suggest the potential of endophytic fungi of *C. amboinicus* as source for antimicrobial agents.

Introduction

The need for searching new bioactive compounds has increased in the field of medicine and industry over the failure of currently used antibiotics. The emergence of new strain of pathogenic microbes possessing resistance to the currently available antibiotics in clinic becomes a growing concern.[1] Various bioactive compounds have been isolated from natural environment including plants as a major source for drug discovery. Many secondary metabolites exhibit antimicrobial properties with some shows antimicrobial activity against multidrug resistant.[2,3] However, despite its complexity in chemical constituents, various factors such as seasonal and geographical specificity, the need for cultivation land and unselective exploitation of medicinal plants for extraction may limits its potential use as source of antimicrobial agents. The finding that endophytes, organisms that colonize healthy plant tissues and could function as alternative source of metabolites, attract more researches to focus on this microorganism for searching novel bioactive compounds having pharmaceutical importance with minor environmental impacts. The fact that various endophytes could synthesize metabolites with antimicrobial potential such as sesquiterpene 1a-10a-Epoxy-7a-hydroxyeremophil-11-en-12,8-b-olide,[4] diterpenes Periconicins A,[5,6] Scoparasin B,[7] steroids,[8] alkaloid Phomoenamide,[9] phenolic compound Colletotric acid,[10] aliphatic compound Brefeldin A[11] and peptide Cryptocandin,[12] these provide evidence the capacity of endophytes as source of bioactive compounds.

Coleus amboinicus Lour. is medicinal plant belong to the family Lamiaceae. Traditionally, this plant is used for treating cough and fever indigestion and loss of appetite,[13] throat infection and nasal congestion,[14] lactagogue,[15] constipation and digestive problems.[16] This plant is reported to have antimicrobial and antioxidant properties.[17-19] Essential oil of this plant is effective against fungi and bacteria;[20,21] whilst hydroalcoholic extract of this plant is active against *methicillin resistant Staphylococcus aureus* (MRSA).[18] The leaves of this plant are shown to have in vivo antimalarial activity against *Plasmodium berghei yoelii*.[22] Flavonoid contents of this plant exhibited antimicrobial activities against *P. aeruginosa, B. subtilis, E. coli, S. aureus, T. mentagrophytes and A. niger*.[23] The leaves of *C. amboinicus* contain phenolic compounds such as carvacrol, flavonoid, rosmarinic, caffeic acid and chlorogenic acid.[24] The flavonoid contents of this plant include flavon salvigenin, 6-methoxygenkwanin, quercetin, chrysoeriol, luteolin, apigenin, flavanon eriodyctol and flavanol taxifolin.[25]

This study is aimed to isolate endophytic fungi from *C. amboinicus* and screen for their antimicrobial activity.

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Materials and Methods

Source and isolation of endophytic fungi

Plant materials were collected from Medicinal Plant Garden, Faculty of Pharmacy, Universitas Gadjah Mada on January 2013. Isolation of endophytic fungi was conducted according to Ding et al. with slight modification.[26] The leaves and stems were washed with running tap water for 10 minutes and surface-sterilized using 70% ethanol for 1 minute, immersed on 5% sodium hypochlorite for 3 minutes and drained. After re-soaking in 70% ethanol for 30 seconds, the samples were rinsed three times in sterile distilled water and surface-dried with sterile filter paper. After surface sterilization the samples were cut aseptically into 1 cm long segments. The surface sterilized leaves and stems were placed onto PDA plates amended with 30 µg/mL streptomycin to suppress the bacterial growth. The plates were incubated at 25°C for 2 to 3 days. The hyphal tip of endophytic fungi growing out from the segments were transferred into new PDA plates amended with 30 µg/mL streptomycin and further incubated for 10 – 14 days. Culture purity was obtained by several times of subculturing. The pure cultures were grown on PDA plates without antibiotics and maintained for culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada. The endophytes were also cultivated for 14 days on potato dextrose broth for investigations of biological activity.

Screening the antimicrobial activity of endophytic fungi

Endophytic fungi isolated from *C. amboinicus* were subjected to screening for antimicrobial activities against six testing microorganisms including *E. coli*, *P. aeruginosa*, *S. thypi*, *S. aureus*, *B. subtilis and C. albicans.* Antimicrobial activity was determined by hole plate assay.[27] Briefly, endophytic fungi grown on PDA were cut out using a sterile cylindrical plug borer (7 mm diameter) and placed onto petri dishes containing testing microorganisms. The testing plates were incubated for 24 hours at 37°C. Antimicrobial activity was determined by the presence of inhibition zone around the endophytic fungi plug and was measured in millimeter (mm).

Fermentation and semipolar extraction of fermentation cultures

The endophytic fungi showed potential antimicrobial activities were fermented in 200 mL culture flasks containing potato dextrose broth for 14 days at 25°C on a shaker at 160 rpm. The mycelia were separated from the fermentation broth by filtering with Whatman filter paper and the broth were further centrifuged at 4000 rpm for 5 min. Liquid supernatant was extracted with an equal volume of ethyl acetate thrice and the ethyl acetate fractions were evaporated to yield an ethyl acetate extract. The extracts were dissolved in ethanol for antimicrobial testing.

Antimicrobial and TLC bioautography testing

The antimicrobial testing was carried out by using disc diffusion method.[28] The extract was dissolved in ethanol and a series dose of extracts (31.25 – 500 µg/disc) were prepared with each consecutive disc containing half amount of the consecutive dose. Kanamycin (10 µg/disc) was used as positive standard and ethanol was used as negative control. The discs containing extracts were placed onto the surface of agar plates containing testing microorganisms and then were incubated for 24 h at 37°C. The inhibition zones were measured in mm at the end of incubation. To characterize the bioactive compounds presence in the extract, contact bioautography was carried out.[27] Components within extracts were separated by thin layer chromatography and the TLC plates containing the separated compounds were placed onto surface of agar plates containing testing microorganisms for 30 minutes. After removing the TLC plates, the agar plates were incubated for 24 h at 37°C. Clear inhibition zone appeared in the place where the separated compounds showed activities against testing microorganisms.

Phytochemical analysis

The ethyl acetate extracts from potential endophytic fungi were analysed for chemical contents using spray TLC visualization detection reagents.[29]

Results

Isolation and primary screening of endophytic fungi

Three strains were obtained from *C. amboinicus*, two of them were isolated from the leaves and one strain from the stem (Figure 1). These isolates were screened for antimicrobial activity against six testing microorganisms. CAL-2 and CAS-1 are effective against *B. subtilis* and only CAL-2 suppressed the growth of *P. aeruginosa*, *S.thypi* and *S.aureus* (Table 1). CAL-1 was found to be active against *E. coli* only. None of the endophytic fungi exhibited inhibition against *C. albicans*. CAL-2 and CAS-1 were found to be more promising for antimicrobial activity and therefore was chosen for further experiments.

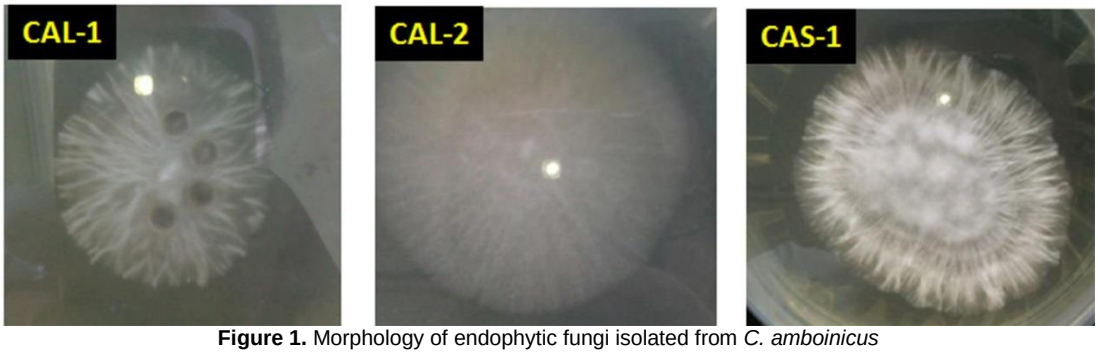

Figure 1. Morphology of endophytic fungi isolated from *C. amboinicus*

Table 1. The presence of clear inhibition zone showed by endophytic fungi by agar plug method

No.	Fungi Code	*E. coli*	*P. aeruginosa*	*S. thypi*	*S. aureus*	*B. subtilis*	*C. albicans*
1	CAL-1	+	-	-	-	-	-
2	CAL-2	-	+	+	+	+	-
3	CAS-1	+	-	-	-	+	-

(+): inhibition zone is present, (-): no inhibition zone

Antimicrobial Activity

CAL-2 and CAS-1 isolates were fermented in potato dextrose broth and the production of secondary metabolites was extracted using ethyl acetate. The ethyl acetate extract production of CAL-2 was around 130-150 mg/ L, whilst the fermentation of CAS-1 produce ethyl acetate extract of around 200-250 mg/L. The CAL-2 crude extract showed inhibition against four testing microorganism with the highest inhibition was exhibited against *P. aeruginosa* (Table 2). At dose of 250 µg, the extract is still able to inhibit the growth of *P. aeruginosa* (7.48 mm) while others showed no inhibition. CAS-1 extract at given concentration inhibited the growth of *B. subtilis* only (Table 3), but at dose of 125 µg, this extract still showed inhibition of 7.1 mm.

Table 2. Inhibition zones of ethyl acetate extract of CAL-2 endophytic fungi as determined by disc diffusion method. Data is representative of at least two independent experiments

No.	Testing microorganisms	Diameter of inhibition zone (mm)						
		Streptomycin (100 µg) – (+) control	500 µg	250 µg	125 µg	62.5 µg	31.25 µg	Ethanol (10 µL) – (-) control
1	*S. aureus*	20.15	8.82	-	-	-	-	-
2	*P. aeroginosa*	25.43	10.95	7.48	-	-	-	-
3	*B. subtilis*	24.45	6.53	-	-	-	-	-
4	*S. thypi*	24.13	7.18	-	-	-	-	-

(-): no inhibition zone

Table 3. Inhibition zones of ethyl acetate extract of CAS-1 endophytic fungi as determined by disc diffusion method. Data is representative of at least two independent experiments

No	Testing microorganisms	Diameter of inhibition zone (mm)						
		Streptomycin (100 µg) – (+) control	500 µg	250 µg	125 µg	62.5 µg	31.25 µg	Ethanol (10 µL) – (-) control
1	*B. subtilis*	25.00	11.00	8.98	7.10	-	-	-
2	*E. coli*	25.00	-	-	-	-	-	-

TLC bioautography analysis

TLC bioautography was conducted to further determine the compounds responsible for antimicrobial activity. Ethyl acetate extract was separated using thin layer chromatography and the TLC profile was examined visually and under UV_{366} and UV_{254} lamp. There is major compound within CAL-2 extract with hRf between 35 – 50 (Figure 2) which absorb UV_{254} light and fluoresce bright blue signal under UV_{366}. This band of major compound showed inhibition against the four testing microorganisms with different characteristics, indicating that this band may consist of multiple compounds. CAL-2 extract also showed inhibition against *B. subtilis* with hRf 80 and against *S. aureus* with hRf 38. TLC bioautography analysis of CAS-1 extract showed two inhibition zones against *B. subtilis* which has hRf of 4 and 95.

Phytochemical analysis

Among detection reagents used for chemical content analysis, four showed positive detections with CAL-2 extract which indicate that the active metabolites may contain chemical groups of terpenoids, phenylpropanoids and phenolic compounds possibly having ketone or aldehydes moiety in their structures (Table 4, Figure 3). The positive reaction of CAS-1 extracts towards anisaldehyde-sulphuric acid and Dragendorff reagents suggested the presence of terpenoids, propylpropanoids, alkaloids or heterocyclic nitrogen compounds.[29]

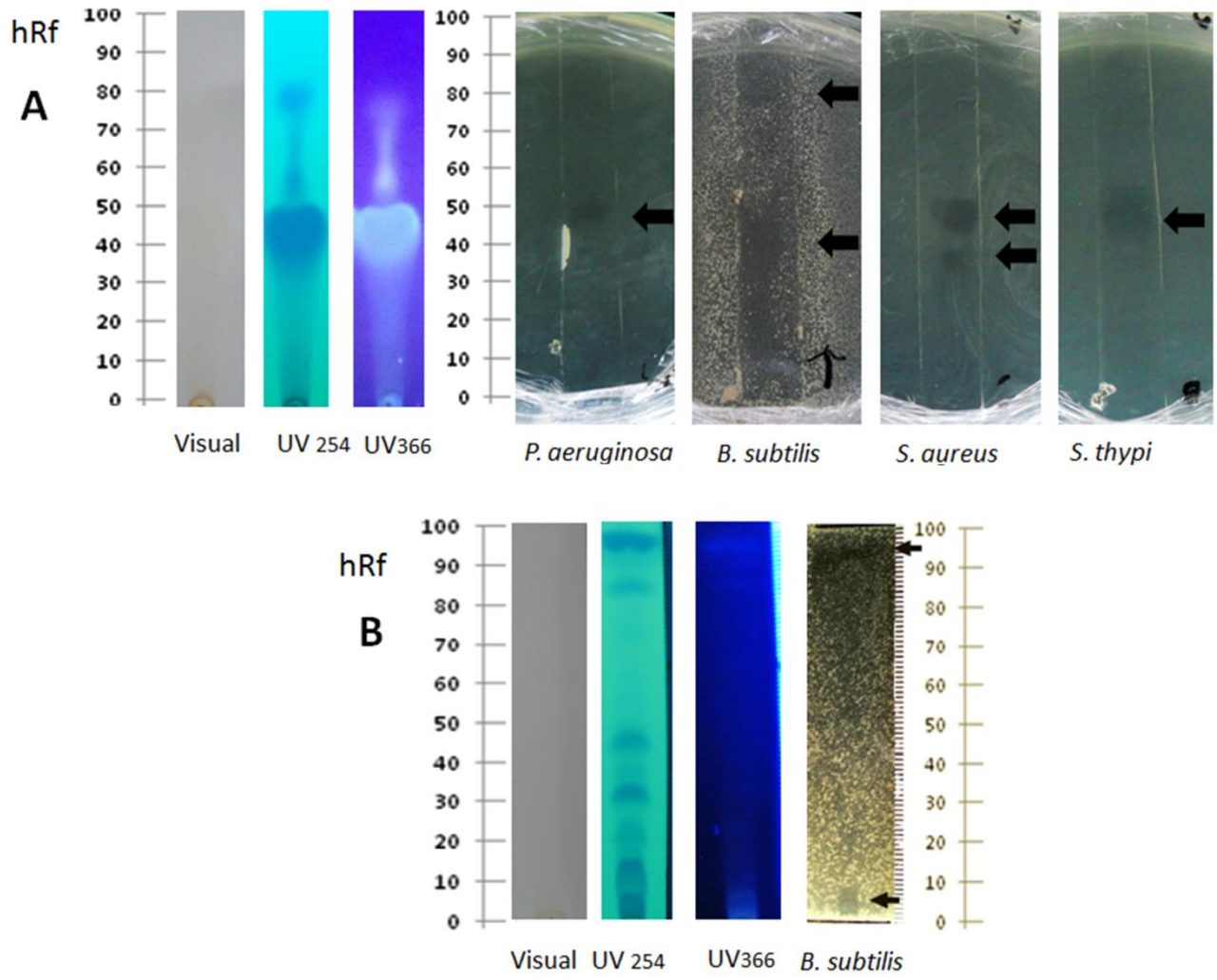

Figure 2. TLC-bioautography profiles exhibited the presence of multiple compounds which have antimicrobial activities. A) The compounds within CAL-2 extract were separated by TLC (stationary phase: silica gel 60 F_{254}, mobile phase = n-hexane: ethyl acetate: methanol (2:6:1); 1 drop glacial acetic acid). TLC plates were placed onto media containing testing microorganisms (*P. aeruginosa, B. subtilis, S. aureus, S. thypi*) for 30 minutes. Following 24h incubation at 37°C, inhibition zones were observed and were correlated with hRf of the bioactive compounds. B) The same methods were applied to CAS-1 extract with stationary phase: silica gel 60 F_{254}; and mobile phase = chloroform: ethyl acetate (7:1). The separated compounds were tested against *B. subtilis*. Dark arrows indicated the presence of antimicrobial compounds.

Table 4. Detection of bioactive antimicrobial compounds using TLC visualization detection reagents

hRf of separated extracts	TLC visualization detection reagents					
	vanillin-H_2SO_4	Anisaldehyde- H_2SO_4	DNPH	$FeCl_3$	H_3BO_3	Dragendorff
CAL-2	-	-	-	-	-	-
35 - 50	brown	n/a	yellowish brown	red	yellow	-
56	brown	n/a	yellow	-	-	-
75	brown	n/a	yellow	-	-	-
CAS-1	-	-	-	-	-	-
4	n/a	violet	-	-	n/a	-
95	n/a	-	-	-	n/a	orange

(-): negative, n/a: not available

Discussion

In our study we found three endophytic fungi which showed inhibition against testing microorganisms with different level of inhibition. One of them (CAL-2) showed broader spectrum than the others. The present study is in line with the earlier findings that endophytic fungi are rich of secondary metabolites having biological activities.[30-33] The endophytic fungi obtained from this study are isolated from medicinal plant *C. amboinicus* which has been traditionally used to treat various diseases.[13-16] The choice of plant as source of endophytic fungi is determined by rationale that the endophytic

fungi may produce compounds which are associated with the plant's ethno botanical history. One of good examples is the discovery of a deoxypodophyllotoxin-producing endophytic fungus harbored in *J. communis*.[34] *Juniperus* plants are known to contain therapeutically important anticancer lignans podophyllotoxin and deoxypodophyllotoxin and has been used since the first century A.D to stop tumors or swelling.[35,36]

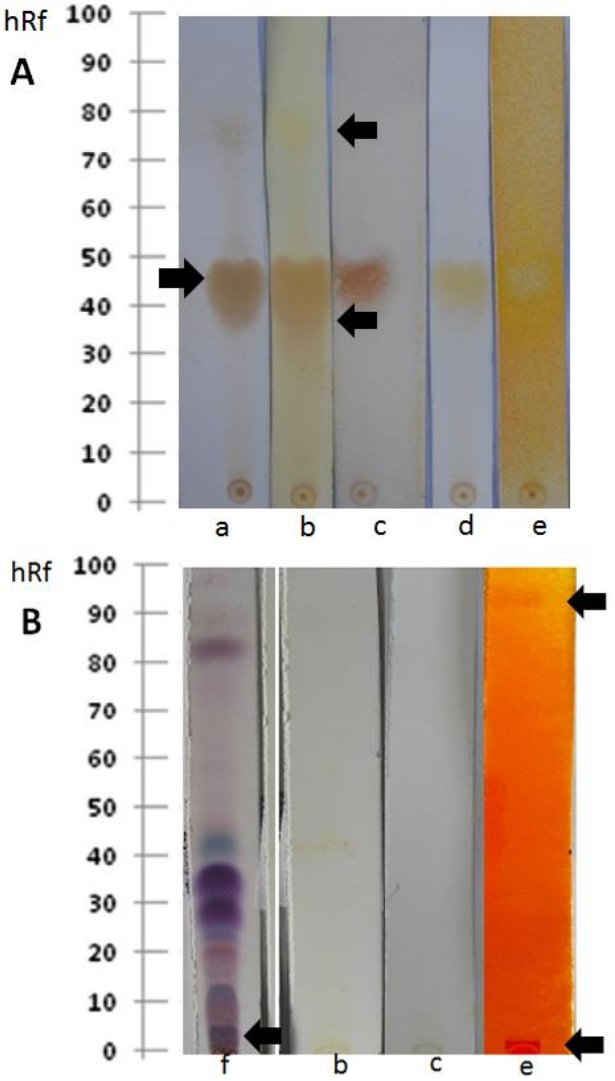

Figure 3. Different compounds are responsible for antimicrobial activities. The extracts were separated by TLC with stationary phase of silica gel 60 F_{254} and mobile phase of n-hexane: ethyl acetate: methanol (2:6:1); 1 drop glacial acetic acid) for A) CAL-2 and stationary phase of silica gel 60 F_{254} and mobile phase of chloroform: ethyl acetate (7:1) for B) CAS-1. The presence of bioactive compounds were visualized using a) vanillin-sulphuric acid, b) DNPH c) $FeCl_3$, d) H_3BO_3, e) Dragendorff, f) anisaldehyde-sulphuric acid. Dark arrows showed the correlated antimicrobial compounds.

Antimicrobial activities of endophytic fungi have been reported by various groups.[26,37-39] The bioactive compounds were found to vary including terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, polyketides, and peptides.[4,9,11,12,33] In this study two promising endophytic fungi having antimicrobial activity were obtained. The chemical constituents within CAL-2 are less than that of CAS-1. However, CAL-2 produces a compound which seems to be major within the extract. Whilst CAL-2 showed broader spectrum of inhibition, CAS-1 is only active against *B. subtilis*. The antimicrobial activity differences could be reflected by the difference in chemical composition between the two extracts which reflects the diversity of metabolites.

TLC bioautography which was conducted to localize and screen the active compounds on chromatogram revealed that CAL-2 extracts showed at least three inhibition zones against four testing microorganisms, whilst CAS-1 extract exhibited two inhibition zones against *B. subtilis*. Detection using TLC visualization reagents suggests the presence of terpenoids, phenyl propanoids and phenolic compounds within CAL-2 extracts, whilst the CAS-1 extract contain terpenoids, propylpropanoids, alkaloids or heterocyclic nitrogen compounds. This data is in line with recent findings that these chemical groups of compounds can be produced by endophytic fungi and have potential to be developed as antimicrobial agents. Sesquiterpene, 10,11-dihydrocyclonerotriol has been isolated from endophytic fungi of medicinal plant *Azadirachta indica* and this compound showed antifungal activities.[40] Similarly, sanguinarine, a quaternary benzo[c]phenanthridine alkaloid has also been produced by endophytic fungi of *Macleaya cordata*, *Fusarium proliferatum* BLH51 and this compound is known to possess antibacterial properties.[41] An antimicrobial compound 4-(2,4,7-trioxa-bicyclo[4.1.0]heptan-3-yl) phenol has been isolated from *Pestalotiopsis mangiferae*, an endophytic fungus associated with *Mangifera indica* Linn.[42] These recent findings suggested that similar chemical groups of compounds may be produced by the endophytic fungi being studied. The differences in the environments where the plants are growing may result in the differences in biodiversity and types of bioactive compounds reside within the endophytic fungi.

Conclusion

The present study suggests that endophytic fungi of *C. amboinicus* may possess antimicrobial potential. Future characterization is needed to elucidate the structure of the bioactive compounds.

Acknowledgments

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Conflict of Interest

The authors declare there is no conflict of interest.

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