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Isolation and antibacterial activity of endophytic fungi from citronella grass (*Cymbopogon nardus*)

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Abstract. *Fais MM, Yurnaliza Y, Hastuti LDS. 2022. Isolation and antibacterial activity of endophytic fungi from citronella grass (*Cymbopogon nardus *(L.) Rendle). Biodiversitas 23: 6564-6569.* Endophytic fungi are a group of functional microorganisms from plants that can be used as a sustainable source to produce bioactive metabolites including pharmaceuticals. Citronella grass (*Cymbopogon nardus* (L.) Rendle) is a kind of medicinal plant that could be used to research the capabilities of endophytic fungi it harbors. The objective of this study was to isolate different endophytic fungi from leaves and stem of citronella plant that may be able to produce antibacterial substances. The bacteria Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* ATCC 25922 were used in plug agar method to determine the potency of endophytic fungi as antibacterials. Using ITS1F and ITS4R primers, endophytic fungal isolates were identified molecularly. Result showed that total 20 isolates of endophytic fungi were obtained from citronella, 15 isolates from stem and 5 isolates from leaves. Three fungal isolates i.e. CNB 254, CNB 253, and CND 111, exhibited highly potent antagonistic activity against tested bacteria. The ethyl acetate extract had a higher antibacterial ability than methanol extract based on the diameter of inhibition zone formed. The three potential isolates were molecularly identified and submitted to GenBank, such as *Trichoderma atrobrunneum* CNB 253 (OP584477), *Trichoderma afroharzianum* CNB 254 (OP584478), and *Fusarium pernambucanum* CND 111(OP584479). The results of this study revealed that citronella grass has a relationship with endophytic fungi that include strains that may be exploited as a source of antimicrobials.

Keywords: Antibacterial activity, *Cymbopogon nardus*, endophytic fungi, *Fusarium, Trichoderma*

INTRODUCTION

Cymbopogon nardus (L.) Rendle (Poaceae) is a medicinal plant used in the fields of cosmetics, perfumery and food industry for its essential oil due to its characteristic and pleasant lemon fragrance (Ranade and Thiagarajan 2015). The major constituents of lemongrass essential oil were α-citral, β-citral, nerol, geraniol, citronellol, terpinolene, geranyl acetate, myrcene, α-terpinol, and other compounds (Haque et al. 2018). The pharmacological activity of lemongrass and its relatives mainly derived from its essential oils other than alkaloids, phenols, and tannins. Several *Cymbopogon* species are used in the formulation of traditional medicine and also have analgesic, anticancer, anti-inflammatory, antioxidant, antiproliferative, antipyretic, cardio-protective, hypolipidemic, hypoglycemic, and neuropharmacological activities (Bayala et al. 2020; Bharti et al. 2013; Bone and Mills 2013; Ekpenyong et al. 2015).

In addition to having advantageous secondary metabolites, medicinal plants also serve as reservoirs for endophytic microorganisms including endophytic fungi. In healthy plant tissues, endophytic fungi are found in intercellular spaces but do not display any harmful effects or disease symptoms (Nazir and Rahman 2018). The presence of endophytic microorganisms in the host plant is mutually beneficial and productive. Endophytes produce an array of bioactive compounds that aid in plant growth, development and resistance to the stressful environment, while plants as a host protects and provide them space (niche) and simple compounds as nutrition for their growth (Nair and Padmavathy 2014). Citronella grass or lemongrass (*Cymbopogon nardus*) is an example of medicinal plant that could be used to investigate the capabilities of endophytic fungi that harbor it (Rahmi et al. 2022). Endophytic fungi may be induced by citronella grass to produce comparable secondary or intermediate metabolites that are derived from the same active constituents in the host. The production of bioactive compounds by endophytic fungi represents a challenging but promising way to supply medicinal plants as raw materials for drug discovery and development (Kaul et al. 2012). In order to reduce the use of citronella plants, some bioactive compounds may be produced by endophytic fungi through fermentation. Compounds produced by fermentation of endophytic fungi also exhibit activity resembling that of host bioactivities, including its antimicrobial properties (Caruso et al. 2022; Ibrahim et al. 2014).

Multidrug resistance (MDR) in microbes has led to a serious health crisis worldwide and is responsible for about 700,000 morbidities annually (Scarafile 2016). There is a need to rediscover ancient therapeutic methods in order to create cutting-edge therapeutic strategies for MDR

pathogens. It is being attempted to investigate therapeutic potential of plant-based compounds or crude extracts from plant-associated endophytic fungi. Despite advance in the use of endophytes across a variety of fields, knowledge of their potential as antimicrobial agents and their mode of action against MDR pathogens is incomplete (Pasrija et al. 2022). Consequently, there are limitations on the use and development of endophytes as potential drug candidates. The purpose of this study was to isolate endophytic fungi from citronella grass and investigate whether endophytic fungi can produce antibiotics or antibacterials that are effective against enteric pathogens and bacteria that are resistant to antibiotics in humans.

MATERIALS AND METHODS

Isolation of endophytic fungi from citronella grass

Isolation of endophytic fungi from citronella was conducted according to the procedure of Rahmi et al. (2022). The plant samples were collected from a local plantation in Pante Kulu Pidie Village, Aceh, Indonesia. The leaves and stems of citronella were cleaned with running water for 20 min. Surface sterilization was performed using 96% EtOH for 2 min, followed by 3% NaOCl for 5 min, and 96% EtOH for 30 secs. Finally, plant samples was rinsed with sterile distilled water twice a times and dried with filter paper. The leaves and stem of citronella were then placed on Potato Dextrose Agar (PDA) media supplemented with chloramphenicol and incubated at room temperature. Fungal colonies growing from plant parts were then subcultured several times on new PDAC media until a single fungal colony was obtained. Pure fungal colonies were observed for their morphological characteristics i.e., colony shape, color, and surface. Microscopical observations were made by observing the fungal mycelium under a compound microscope at 100× magnification.

Antagonistic test of endophytic fungi from citronella grass

Dual culture assay was used to test the antibacterial ability of endophytic fungi against MRSA (Methicillin-resistant *Staphylococcus aureus*) and *Escherichia coli* ATCC 25922. Bacterial suspensions were made with a cell density equivalent to a 0.5 McFarland solution (10^8 CFU/mL) and swabbed on the surface of Mueller Hinton Agar (MHA) media using a sterile cotton swab. The seven days old mycelium of endophytic fungi was grown on PDA medium and cut with a cork borer (5 mm diameter). The disc was then placed on MHA media containing MRSA and *E. coli* ATCC 25922. The culture was incubated at room temperature for 24 hours. Bacterial growth activity was indicated by the presence of a clear zone around the fungal hyphae. The clear area was measured using a digital caliper and expressed as the average diameter of the inhibition zone (Maesaroh et al. 2020).

Fermentation and crude extraction of endophytic fungi from citronella grass

Solid-state fermentation was used to produce a large amount of fungal biomass and metabolites. Endophytic fungi that showed inhibitory activity against the indicator bacteria were then re-cultured in a PDA medium for ten days. The endophytic fungal mycelium was then cut into pieces and placed in two flasks (250 mL) wrapped in an aluminium foil to avoid photolysis. Each flask containing pieces of fungal mycelium, was immersed with 70% MeOH and 70% EtOAc as solvents until the mycelium pieces were submerged, then incubated for 72 hr with an orbital shaker. The MeOH and EtOAc extracts were separated from the mycelium by filtering with filter paper and then drying in an oven at 70°C for 5 minutes. Concentrated crude extract was used for further analysis.

Antibacterial test of fungal crude extract

Disc diffusion method was employed to determine the antibacterial activity of fungal crude extracts.The crude extracts were prepared into various concentrations (w/v) i.e. 100%, 50%, and 25%, using 10% DMSO as solvent. The indicator bacteria were rejuvenated for 24 hr. The bacterial suspensions were prepared with a cell density equivalent to a 0.5 McFarland solution (10^8 CFU/mL). Each bacterial suspension was placed on the solid surface of MHA media by using a sterile cotton swab. A six-mm blank disk already impregnated with different concentrations of MeOH and EtOH was placed on the surface of MHA medium containing test bacteria. As a comparison, a negative control was used in the form of 10% DMSO, MeOH, and EtOAc. Ciprofloxacin (5 g), a standard antibiotic was used as a positive control. The culture was incubated at room temperature for 24 hr. The presence of clear zone around discs indicated the antibacterial activity of crude extracts, antibiotics and solution.The clear area was measured using a digital caliper and expressed as the average diameter of the inhibition zone. The effective percentage of an extract was compared to the standard antibiotics (ciprofloxacin) by using the following formula (Ghasemi et al. 2003):

$$Antimicrobial\ index = \frac{Inhibition\ zone\ of\ sample\ (mm)}{Inhibition\ zone\ of\ standard\ antibiotics} x100\%$$

Identification of potential fungal strains

Fungal genomic DNA was isolated, amplified with ITS1F and ITS4R primers, and sequenced using a DNA sequencer by Macrogen Inc., Singapore. The DNA sequences obtained were then analyzed for genetic similarity using the GenBank database from NCBI using the Basic Local Alignment Search Tool for the nucleotide sequence program (BLASTn). The phylogenetic tree was constructed using MEGA11 software (Tamura et al. 2021). The DNA sequences were aligned using the ClustalW method by comparing the fungal DNA sequences from the BLASTn results, which had the closest DNA sequence resemblance to the endophytic fungi to be identified. After the DNA base sequences were aligned, then a phylogenetic

tree was created using the Neighbor-Joining feature with 1000× bootstrap repetitions.

RESULTS AND DISCUSSION

The stems and leaves of *C. nardus* were colonized by endophytic fungi. A total of 20 fungal isolates were isolated from the samples with a higher proportion from leaves (15 isolates) than in stem (5 isolates). The morphology of each isolate is presented in Figure 1 and Table 1. Our finding is similar to a study by Deshmukh et al. (2010) who isolated more fungal isolates from leaves than stem of *Cymbopogon citratus*. According to Carroll (1988), grass endophytes may be originated from exposed aerial parts of the host plants to air and water droplets, which may also account for a higher number of fungal species in leaves compared to stem.

Three potential isolates, namely CNB 253, CNB 254, and CND 111, inhibited the growth of MRSA. Meanwhile, *Escherichia coli* ATCC 25922 was only inhibited by one potential isolate namely CNB 254. A clear zone without a bacterial overgrowth developed around the endophytic agar plug placed on MHA media. This zone of inhibition shows that indicator bacterial growth was inhibited by the presence of bioactive substances produced by the endophytic fungi of citronella. Therefore, the three isolates were investigate further for their antibacterial metabolites. In general, the EtOAc extracts displayed a greated inhibition towards MRSA and *E. coli* than the MeOH extracts (Figure 2).

Figure 1. Colonies of endophytic fungi isolated from the leaves and stems of citronella grass (*Cymbopogon nardus*) grown on PDA medium after 14 days

Table 1. Morphological characteristics of endophytic fungi from citronella grass (*Cymbopogon nardus*)

Isolates	Form	Color	Surface
CNB221	Filamentous	White beige	Umbonate
CNB212	Filamentous	Purplish white	Umbonate
CNB253	Filamentous	Green young	Raised
CNB211	Filamentous	Green	Raised
CNB254	Filamentous	Dark green	Raised
CND211	Irregular	Black	Raised
CND251	Irregular	Whitish black	Raised
CND151	Irregular	Black	Raised
CND111	Filamentous	White	Raised
CND252	Filamentous	Yellowish white	Raised
CND134	Filamentous	Blackish white	Umbonate
CND153	Filamentous	Green	Raised
CND141	Filamentous	Brownish white	Raised
CND112	Filamentous	Black	Umbonate
CND212	Filamentous	Greenish white	Raised
CND133	Filamentous	White	Raised
CND243	Filamentous	White cream	Raised
CND253	Filamentous	Brown, black	Raised
CND254	Filamentous	White	Raised
CND242	Filamentous	White	Raised

Different diameters of inhibition zone were produced by using endophytic fungi from MeOH and EtOAc crude extracts. MeOH and EtOAc extracts from endophytic fungi produced inhibition zones with varying diameters. The 100% EtOAc CNB 254 extract had 29.5 mm highest inhibition zone against MRSA. According to Avinash et al. (2015), EtOAc extract of endophytic fungus from *Cymbopogon caesius* inhibited the growth of several pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella enterica*, and *Xanthomonas campestris*. Mohamed and Alat (2020) also reported that EtOAc extract of an endophytic fungus from *Cymbopogon* sp. inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* sp. As a positive control, the second-class quinolone antibiotic ciprofloxacin can prevent the growth of the majority of gram-positive and gram-negative aerobic bacteria as well as some anaerobic gram-negative bacteria. As a result of ciprofloxacin's mechanism, which involves inhibiting the enzymes topoisomerase II (DNA gyrase II) and topoisomerase IV during cell division, which is required to replicate DNA, bacterial growth activity is interfered with (Fair and Yitzhak 2014).

The diameter of the inhibition zone was larger when EtOAc was used as a solvent extract than MeOH. MeOH is a polar solvent that can dissolve nearly all classes of organic compounds, whether they are polar, semi-polar, or non-polar. In contrary, EtOAc solvents are frequently used in the extraction of endophytic fungal cultures because this solvent is semi-polar and can extract the components present in endophytic fungal cultures (Rahmawati et al. 2018). Endophytic fungal cultures were more effectively extracted by EtOAc solvent than by any other solvent. This is consistent with research by Desale and Bodhankar (2013), who found that *E. coli, S. typhimurium, B. cereus, B. subtilis, K. pneumoniae*, and *S. aureus* significantly inhibited by endophytic fungal extract of *Vitex negundo* when it was dissolved in EtOAc, MeOH, and hexane. In addition, Rumidatul et al. (2021) noted that when tested on *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli*, EtOAc extract from the endophytic fungus *Falcataria moluccana* culture demonstrated the highest antibacterial inhibition in comparison to MeOH and hexane extract. The antibacterial index of crude extracts against MRSA and *E. coli* is presented in Table 2. According to Figures 3 and 4, each MeOH and EtOAc extract of endophytic fungus *C. nardus* successfully hindered the growth of indicator bacteria based on the antibacterial index value. With the exception of MeOH CNB 253 extract, which had an antibacterial index value of 88 percent at 100% MeOH extract and EtOAc concentration, all antibacterial index values were 100%. The 25% MeOH CND 111 extract had the lowest antibacterial index value of 41%. The antibacterial index value increases with the concentration of endophytic fungal extract. Antibacterial activity was also observed in the crude extract of endophytic fungi from *Nyctanthes arbor-tristis*, a medicinal plant from India as reported by Gond et al. (2012). The percentage of inhibition was 100% using ciprofloxacin against all tested bacteria with 62.5% of activity from *Colletotrichum dematium* and *Chaetomium globosum* (Gond et al. 2012).

Based on the phylogenetic construction of ITS-rDNA sequence between fungal isolates and GenBank database, identity of each isolate was confirmed likewise CNB 253 as *Trichoderma atrobrunneum* (OP584477), CNB 254 as *Trichoderma afroharzianum* (OP584478), and CND 111 as *Fusarium pernambucanum* (OP584479) as presented in Figure 5. Fungal endophytes may originate from the environment of host plants, which contains microorganisms, fungi, and other trophic components and metabolic processes (Sasse et al. 2017).

Endophytic fungi prefer horizontal transmission for colonizing tissues above the ground. The fungal endophytes spread from one plant to another through spores, biotic elements like herbivores or insects, and abiotic elements like wind or rain, proving that different host plants can harbor different fungi endophytes (Wiewiora et al. 2015). The species diversity and assemblage of endophytic fungi from *Cymbopogon* spp. have been reported from various studies following their bioactivities.

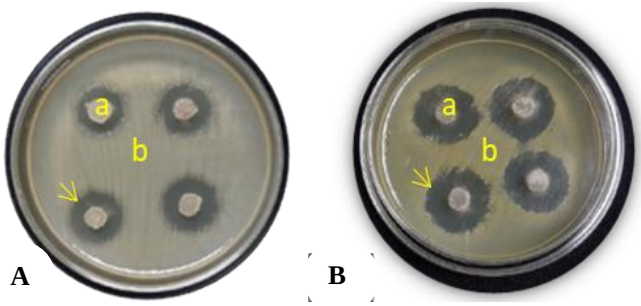

Figure 2. Antibacterial activity of endophytic fungal isolates of citronella grass against (A) *Escherichia coli* ATCC 25922 and (B) MRSA using dual culture assay between (a) agar plug of fungal isolates and (b) bacterial lawn. Arrows show clear zones as an indication of antibacterial activity

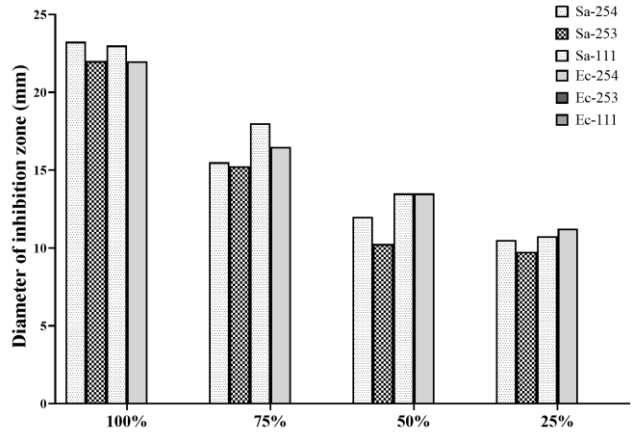

Figure 3. Antibacterial activity of MeOH extract of endophytic fungal strains (254, 253, 111) against MRSA (Sa) and *E. coli* ATCC 25922 (Ec)

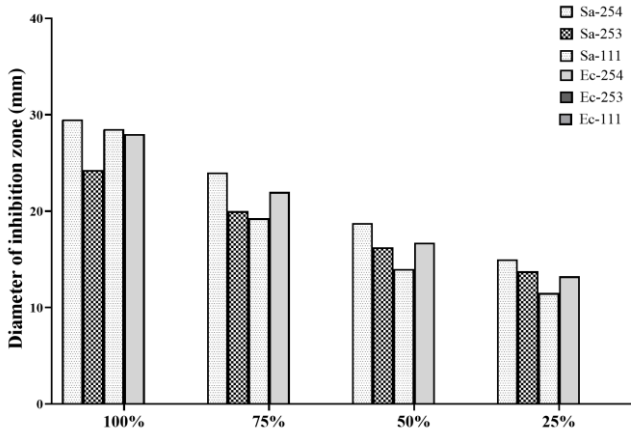

Figure 4. Antibacterial activity of EtOAc extract of fungal strains (254, 253, 111) against MRSA (Sa) and *E. coli* ATCC 25922 (Ec)

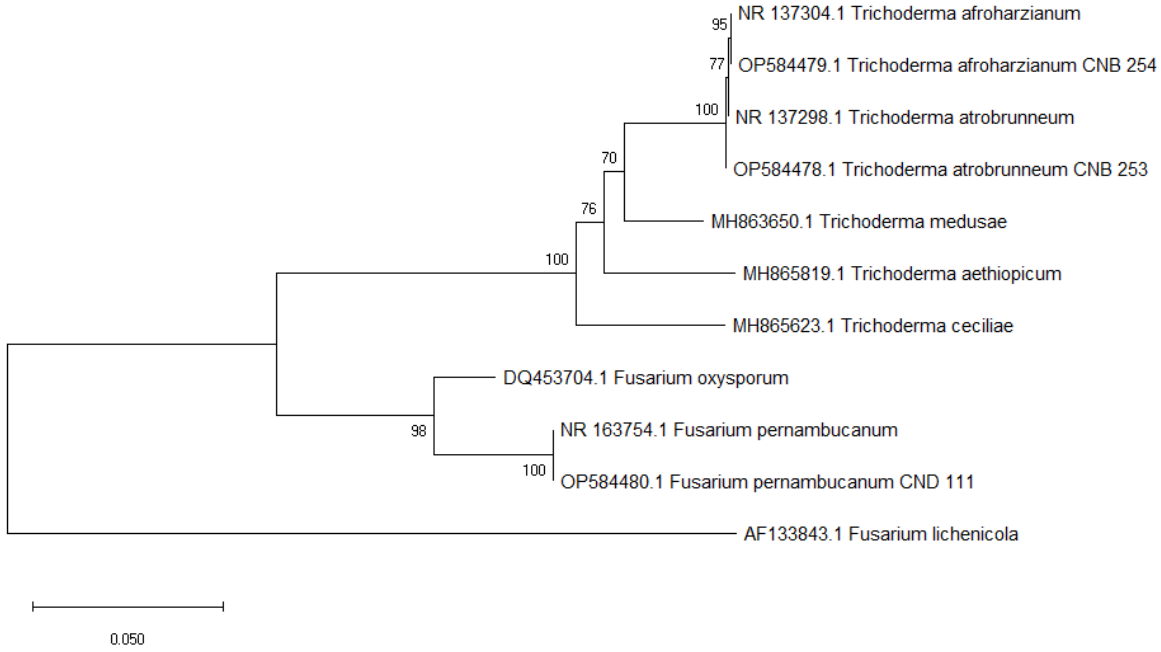

Figure 5. Phylogenetic construction of ITS-rDNA sequence from fungal isolates and GenBank database using Kimura-2 parameter method and bootstrapping with 1000× repetitions

Table 2. Antibacterial activity of crude extracts in the form of antibacterial index value (%) against MRSA and *E. coli*.

Bacteria	Extracts code	Antimicrobial index			
		100%	75%	50%	25%
MRSA	CNB 254 MeOH	100	70	55	48
	CNB 253 MeOH	100	69	51	44
	CND 111 MeOH	100	82	61	41
	CNB 254 EtOAc	100	100	85	68
	CNB 253 EtOAc	100	91	74	63
	CND 111 EtOAc	100	87	64	52
E. coli	CNB 254 MeOH	88	66	54	45
	CNB 254 EtOAc	100	88	67	53

Prior to this study, Rahmi et al. (2022) reported a collection of endophytic fungi i.e. a strain of *Trichoderma* sp., *Nigrospora* sp., and an unidentified fungus from the leaf and stem part of *C. nardus* capable of producing lovastatin, a cholesterol-lowering agent. The presence of *Trichoderma* in plant tissue may be explained from its entry through soil and rhizosphere. Shaikh et al. (2017) reported that rhizospheric soil samples from *Cymbopogon citratus* plantation were inhabited by various members of *Trichoderma*, in which a strain of *Trichoderma viride* was able to produce citral, a potential bioactive compound. In addition, the presence of *Fusarium oxysporum* was also documented in the leaf blade and rhizome of *C. citratus* (Deshmukh et al. 2010).

In conclusion, a total of 20 isolates of fungi were isolated from citronella stem and leaves (five from stems and fifteen from leaves). Three isolates, namely CNB 254, CNB 253, and CND 111 were selected as potential endophytic fungi. Methanol and ethyl acetate extracts were used in disc diffusion method to test their inhibitory potential against MRSA (Methicillin-resistant *Staphylococcus aureus*) and *Escherichia coli* ATCC 25922. The endophytic fungal isolate extract of citronella produced a higher inhibition zone using ethyl acetate as a solvent than methanol. Two isolates were identified as members of *Trichoderma* and one isolate as a member of *Fusarium*.

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