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Research Paper

Antibacterial and Antioxidant Activity of Endophytic Fungi Extract Isolated from Leaves of Sungkai (*Peronema canescens*)

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Abstract
Sungkai is a plant that is widely found in Indonesia. This plant is often used in traditional medicine so the biotechnology of endophytic fungi is highly needed due to natural resources from plants that have been reduced. This study reported on endophytic fungi found in sungkai leaves and the compound produced. Morphological and molecular identification through phylogenetic tree analysis was carried out to determine the endophytic fungal species found. Potato Dextrose Broth media was used for the cultivation process. Ethyl acetate was used as a solvent for the extraction and the evaporation process used a rotary evaporator. Antioxidant and antibacterial tests were carried out using the DPPH method and paper disc diffusion. Chromatographic techniques were used to isolate the compound and spectroscopic analysis was performed to identify its chemical structure. The results of the morphological and molecular analysis showed *Trichoderma asperellum* as an endophytic fungus identified. The pure compound obtained from this endophytic fungus was 4-hydroxybenzoic acid. The antioxidant and antibacterial activity showed a strong category (IC50 = 43.88 μg/mL; MIC 64 μg/mL). This compound was very likely to be a raw material for new antibiotics and antioxidants through further research with various modifications.

Keywords
Bioactive Compound, Endophytic Fungi, *Trichoderma asperellum*, Sungkai

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1. INTRODUCTION

During the Covid-19 pandemic, sungkai leaf is a plant that is believed by the community, especially in South Sumatra (Indonesia), to increase immunity. The secondary metabolites contained in the sungkai plant, namely flavonoids, peronemin, isopropanol, betulinic acid, peronemin, and sitosterol are efficacious as antioxidants and antibacterials in which this bioactivity mechanism can increase the immune system (Dillasamola et al., 2021; Latief, 2021). However, the cultivation of medicinal plants faces many obstacles that reduce plant populations and exploration is needed to find new sources of raw materials for medicinal needs, such as biotechnology of endophytic fungi.

The biotechnology of endophytic fungi is a technology that uses fungi living on plant tissues without harming their hosts (El Hawary et al., 2020; Mbilu et al., 2018). The interesting variety of chemical structures and bioactivity make endophytic fungi a promising focus for natural products (Tiwari and Bae, 2020; Wen et al., 2022). Studies have revealed that endophytic fungi produce secondary metabolites with diverse bioactivities such as antibiotics, antiprotozoals, antivirals, antidiabetics, antiparasitics, anticancers, antioxidants, and immunomodulatory compounds so that endophytic fungi are referred to as secondary metabolite stores (Khan et al., 2019; Manganyi and Ateba, 2020). *Trichoderma* is a genus of endophytic fungi that have diverse bioactivity (Morais et al., 2022; Zhang et al., 2021).

Trichoderma has a good adaptive ability to the environment and its growth rate is much faster. *Trichoderma* can produce various secondary metabolites, such as isonitrile, diketopiperazine, sesquiterpenes, polyketides, alkylpyrone, and peptaibol (Khan et al., 2020; Wu et al., 2017). It has proven that 6-pentyl-α-pyron, which is produced by *T. harzianum* and *T. hamatum*, efficiently has antioxidant and antibacterial properties against *Acidovorax avenae*, *Erutimcarafavora*, and *Xanthomonas campestris* (Al Rajhi et al., 2022; Baazeem et al., 2021). *T. asperellum* is

also known to contain compounds such as alkaloids, tannins, phenolics, triterpenoids, and flavonoids which effectively have bioactivity, such as antipyretic, antibacterial, anticancer, and antioxidant (Gu et al., 2022; Karuppiah et al., 2019; Scudeletti et al., 2021; Singh et al., 2021). Secondary metabolites from endophytic fungi can be used as relevant sources of raw materials for new medicine. Research shows that the bioactive metabolites obtained have a unique chemical structure. These endophytic fungi are able to produce similar metabolites or new compounds that are different from their host (Cruz et al., 2020; El Hawary et al., 2020). This event is a potential that can be developed from this group of microbes for the discovery of new drugs.

2. EXPERIMENTAL SECTION

2.1 Sample Preparation and Isolation of Endophytic Fungi

The sample obtained was identified at the Plant Systematics Laboratory, University of Sriwijaya with number 302/UN9.1.7 /4/EP/2021. The fresh leaves used were in the fourth position from the primary branch. The surface of the leaves was first sterilized before the isolation of the endophytic fungi by washing it with water for ± 5 minutes. Next, the sample was immersed in alcohol 70% for ±3 minutes, rinsed with sterilized-distilled water for ±1 minute, and immersed in 3% NaOCl solution for 1 minute. For the inoculation, the sample was first cut aseptically ± 3 x 1 cm before being inoculated into a petri dish containing PDA. The inoculants were incubated for 3-14 days at room conditions. Purification of fungi endophyte by transferring the colonies to a new petridish containing media and incubating at room temperature for 48 hours (Setiawan, 2022; Hapida et al., 2021).

2.2 Characterization and Identification of Fungal Endophytes Morphologically

Phenotypic characters were used to identify endophytic fungi. The slide culture method was used to observe microscopic characteristics with 1000X in magnification. The phenotype characteristics (macroscopic and microscopic) that emerged were then compared with several references (books and journals) for identification requirements (Pitt and Hocking, 2009;Walsh et al., 2018; Watanabe, 2002).

2.3 Molecular Identification of Fungal Endophytes

Molecular identification was carried out based on the endophytic fungal isolates with the most potential bioactivity. The identification used the ITS DNA (rDNA) area. The amplification process used primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'). The sequences are included in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, the CLUSTAL W method (in the MEGA11 program) was used to align the sequences and the Neighbour-joining tree method was used to construct a phylogeny tree with a bootstrap value of 1000 (Tamura et al., 2013).

2.4 Cultivation and Extraction

Cultivation was carried out by placing 6 blocks of agar (6 mm in diameter) the pure culture of each endophytic fungal isolate obtained was placed in 300 mL Potato Dextrose Broth media. The isolate was cultivated in 15 glass bottles (volume 1 L). Then, the cultures were incubated for 30 days statically at room condition. The medium was separated from the fungal biomass using filter paper and the solvent ethyl acetate was added to the culture medium with the ratio of 1:1. After 10 days, the extracts were separated using a rotary evaporator (Habisukan et al., 2021).

2.5 Antioxidant Activity Test

The antioxidant activity used the DPPH method based on Baliyan et al. (2022), that was 0.2 mL of each extract concentration was added 0.5 mM DPPH solution volume 3.8 mL. The blend solution was incubated in a dark tube for 30 minutes. The absorbance was measured at 517 nm using a spectrophotometer and ascorbic acid was used as a standard. The percentage of inhibition and IC50 value were calculated to determine antioxidant activity (Abbas et al., 2021).

$$\%\text{Inhibition} = \frac{A_k - A_s}{A_s} \times 100\% \quad (1)$$

Ak = Control
As = Samples

2.6 Antibacterial Activity Test

The test of antibacterial activity used the paper disc diffusion method. The media used is MHA (Muller Hinton Agar). The test bacteria used were *Escherichia coli* InaCCB5, *Salmonella typhi* ATCC1048, *Bacillus subtilis* InaCCB120, and *Staphylococcus aureus* InaCCB4. The blank disc paper was dripped with the concentration of 256, 128, 64, 32, 16, 8, and 4 μg/mL to test the Minimum Inhibitory Concentration (MIC). The MIC value showed <100 μg/mL belonged to strong antibacterial (Ding et al., 2019).

2.7 Isolation and Identification of Compound

The most potential extract was prepared by preabsorption and put into the chromatographic column. Next, the extract was eluted by using eluent upon increasing polarity. The eluate was collected to a 10 mL vial and a TLC test was carried out to be grouped into column fractions. Pure compounds were obtained through the process of evaporation, separation, and purification of column fractions with chromatographic techniques. The compound structure identification used 1D and 2D NMR spectroscopy method which included ^{1}H-NMR, 613C-NMR, HMQC, HMBC and compared with the NMR data of the same compound from reference.

3. RESULT AND DISCUSSION

3.1 Isolation and Identification of Fungal Endophytes

This research was the continuation by re-isolating SD8 from sungkai leaves at the fourth position from the primary branch,

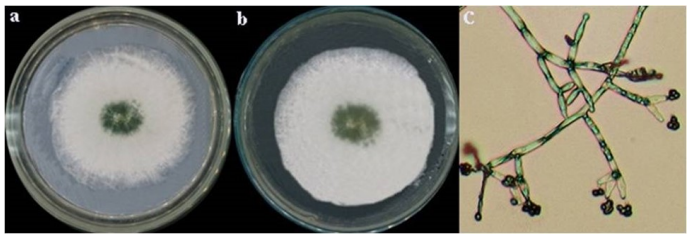

Figure 1. Morphology of *T. asperellum* Found in Sungkai Leaves (a. Front View; b. Reverse View; c. Microscopic Characteristic)

a different position from previous studies. Furthermore, morphological and molecular identification was carried out on SD8 isolate. Macroscopic characteristics showed colonies with white greentint, cottony, umbonate, and radiate while microscopic characteristics showed conidiophores hyaline, short and thick phialides, and globose. Colony turned green with the age (Figure 1).

Molecular identification showed that the isolate SD8 was Trichoderma asperellum with 100% similarity. The phylogenetic tree can be seen in Figure 2 with the sequence as follows:
TGCGGAGGGATCATTACCGAGTTTACAACTCCCAAA
CCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGG
GGTCACGCCCCGGGTGCGTCGCAGCCCCGGAACCAG
GCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTA
GTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCA
AAAATTCAAAATGAATCAAAACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTA
TTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAAC
CCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCG
GGACCCCTCACACGGGTGCCGGCCCCGAAATACAGT
GGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTT
GCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTC
CGTAAAACACCCAACTTTCTGAAATGTTGACCTCGG
ATCAGGTAGGAATACCCGCTGAACTTAAGCATAT

Trichoderma asperellum is a type of fungus that is highly adaptive to the environment. The growth rate tends to be faster than other fungi so that they can fight with nearby pathogens for defined space and nutrients (Stracquadanio et al., 2020; Tyśkiewicz et al., 2022; Zin and Badaluddin, 2020). Therefore, this fungus is often used as a biocontrol of pathogenic fungi in the environment. Research reveals that *T. asperellum* can be found in all parts of the plant because it is easy to spread and no research evidence reports on the specific tissue where it grows (El_Komy et al., 2015; Lahlali et al., 2022). Endophytic fungi isolated from plants, especially medicinal plants, usually have good biological activity. The ability of endophytic fungi to synthesize secondary metabolites is used by host plants for defense and can be used as alternative raw materials for medicines. Research on *T. asperellum* from other

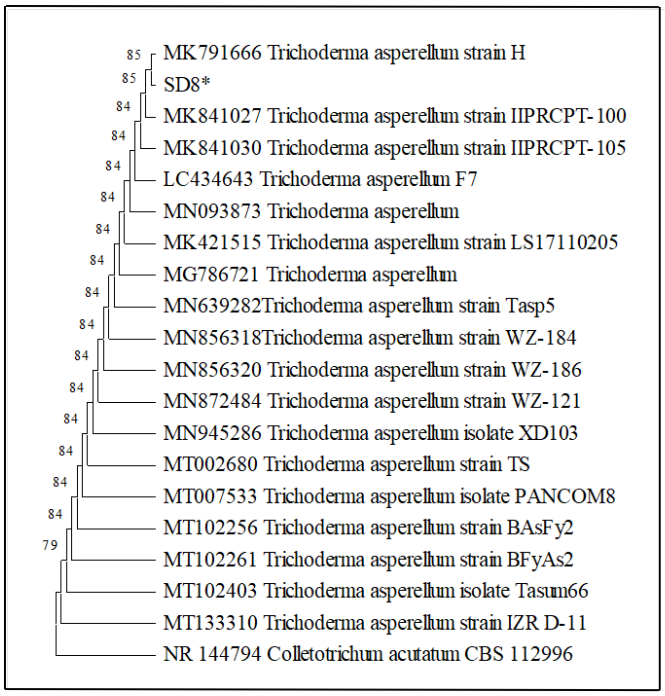

Figure 2. Phylogeny Tree of SD8* Constructed by Using Neighbour-Joining with Bootstrap of 1000

plants has been carried out to reveal its secondary metabolites which have anti-inflammatory, antimicrobial, and antioxidant activities (Sharma et al., 2021; Singh et al., 2021).

3.2 Bioactivity of Fungal Endophyte

The antioxidant and antibacterial activity of ethyl acetate extract and pure compound of *T. asperellum* compared with ascorbic acid and tetracycline as standard can be seen in Table 1.

Table 1 shows the results of the antioxidant and antibacterial properties tests of the ethyl acetate extract of the endophytic fungus *T. asperellum*, and its compound. The results showed that the compounds obtained had strong antioxidant activity (IC50 < 100 μg/mL) and strong antibacterial activity contrary to all test bacteria (MIC ≤ 64 μg/mL). These results indicate that the compounds obtained have the potential to be developed into new materials for pharmaceuticals.

The ethyl acetate extract of *T. asperellum* showed moderate antibacterial activity against *E. coli* and *S. aureus* (MIC = 128 μg/mL) and a strong category for *S. thypi* and *B. subtilis* (MIC ≤ 64 μg/mL). Its antioxidant activity also showed strong activity (IC50 < 100 μg/mL). The metabolites contained in the crude extract of *T. asperellum* were the reason for its good bioactivity. Studies have revealed that secondary metabolites contained in *T. asperellum* are tannins, alkaloids, triterpenoids (1.11 mg/mL), phenolics (5.98 mg/mL), and flavonoids (3.76 mg/mL) (Ikram et al., 2019; Omomowo et al., 2020; Sumilat et al., 2022). Phenolates are the most dominant secondary metabolites found in *T. asperellum*. Phenolic compounds are able to inhibit bacterial growth and reduce oxidative stress. The hydroxyl group

Table 1. MIC and IC50 Values of Ethyl Acetate Extract and Pure Compounds from the Endophytic Fungus *T. asperellum* with Tetracycline and Ascorbic Acid as Standards

Samples	MIC Values (μg/mL)				Antioxidant Activity
	E.-coli	*S.-aureus*	*S.-thypi*	*B.-subtilis*	IC50 (μg/mL)
EtOAc Extract	128	128	32	64	12,2****
Compound	32	64	32	64	43,88***
Tetracycline [a]	4	4	4	4	
Ascorbic Acid [b]					10,08****

Note: [a]Antibacterial standard; [b]Antioxidant standard; antioxidant activity IC50 (μg/mL): **** very strong < 20μg/mL ***strong < 100 μg/mL; **moderate 100-500 μg/mL;*weak > 500 μg/mL

Table 2. The NMR Data of Compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in CD_3OD) and 1* (^{13}C-75.5 MHz in DMSO-d6)

δ_C ppm 1	Type of C 1	δ_H ppm ($\sum$H. Multiplicity (Hz)) 1	HMBC 1	δ_C ppm 1*
136.3	C			127.5
130.6	CH	8.26 (1H, d, J= 9.0 Hz)	130.6; 150.6; 166.3	131.1
123.2	CH	8.30 (1H, d, J= 9.0 Hz)	123.2; 136.3; 150.6	114.4
150.6	C			159.9
123.2	CH	8.30 (1H, d, J= 9.0 Hz)	123.2; 136.3; 150.6	114.4
130.6	CH	8.26 (1H, d, J= 9.0 Hz)	130.6; 150.6; 166.3	131.1
166.3	COOH			169.4

* Cho et al. (1998)

and long saturated side chain give this compound the ability to thwart the protein and DNA synthesis activity of bacteria through inhibition of ribonucleic acid reductase activity, and reduce the surface permeability of bacterial cell walls (Adamczak et al., 2019; Burel et al., 2021). Based on the literature, the constituent of secondary metabolites contained in the extract of endophytic fungi is similar to the host plant. This indicates that endophytic fungi can copy secondary metabolites of their host plants because of their role in mutualistic interactions.

3.3 Compound Isolation and Identification

Ethyl acetate extract of *T. asperellum* (2 g) after being preabsorbed with silica gel in a ratio of 1:1, followed by the separation of compounds on column chromatography using silica gel as a stagnant phase. The eluent used was n-hexane:EtOAc (10:0 → 0:10) to EtOAc:Methanol (10:0 → 0:10) gradient, which obtained five subfractions (F1-F5). Based on the staining pattern on TLC, the F3 subfraction was column chromatographic again with n-hexane:EtOAc (3:7 → 0:10) eluent and gave four subfractions (F3.1-F3.4). Subfraction F3.3 was rinsed with n-hexane:EtOAc (1:1) solvent to obtain compound 1 (31.4 mg).

The ^{1}H-NMR spectral (Figure 3) of the pure compound 1 showed the presence of two proton signals, namely at δ_H 8.26 and 8.30 ppm. both signals have a complicated doublet multiplicity with the integration of two protons. Both of these signals are in the chemical shift of the aromatic region and each has an ortho-lot constant (J = 9 Hz) which demonstrates

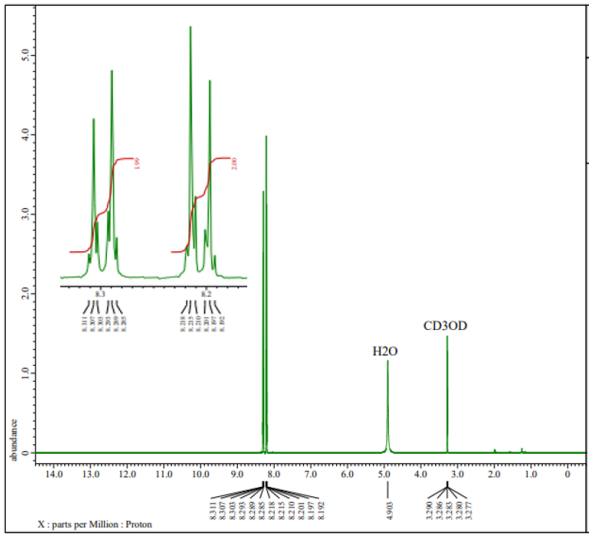

Figure 3. The ^{1}H-NMR Spectre of Compound 1 (^{1}H-500 MHz; in CD_3OD)

that the pure compound is an aromatic compound with para position substitution. This causes two pairs of ortho protons to exist in the same chemical shift.

The ^{13}C-NMR spectra (Figure 4) of the pure compound 1 exhibited the existence of five carbon signals, all of which were sp2. Carbon at δ_C 123.2 and 130.6 ppm with large intensity

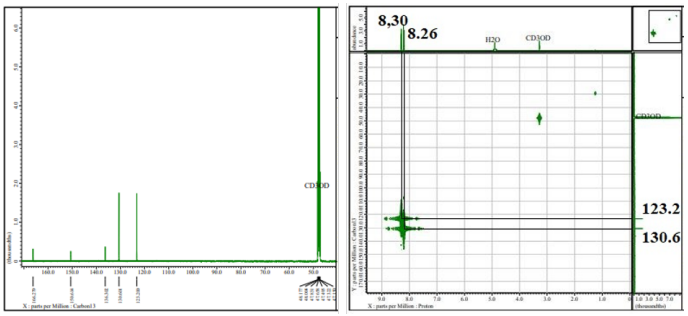

Figure 4. The ^{13}C-NMR and HMQC Spectra of Compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in CD_3OD)

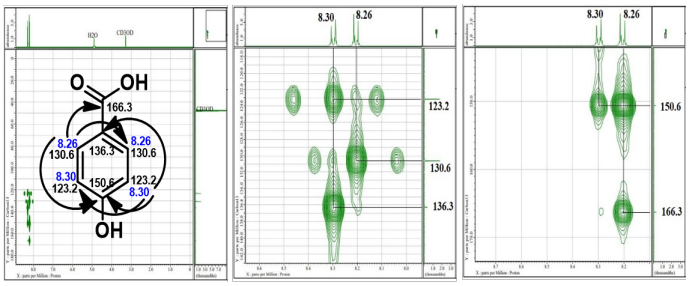

Figure 5. The HMBC Spectra of Compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in CD_3OD)

indicate that there are two carbon equal in each of these signals. This is backed by the ^{1}H-NMR spectrum which shows the presence of two equivalent protons. In the spectrum there are carbons in the low field δ_C 166.3 and 150.6 ppm which indicate the presence of acid carbonyl carbon and oxyaryl carbon, respectively. The analysis of the proton and carbon NMR spectra was confirmed by the data on the HMQC spectrum listed in Table 1, which showed that there were two ^{1}H-^{13}C correlations through one band consisting of two interactions on the aromatic ring.

The HMBC spectre (Figure 5) demonstrated a correlation of ^{1}H-^{13}C through two or three bonds. The aromatic proton signal at δ_H 8.26 ppm is correlated through three bonds with its equivalent aromatic carbon (δ_C 130.6 ppm); oxyaryl quaternary carbon (δ_C 150.6 ppm); and carbonyl carbon of the carboxylic acid (δ_C 166.3 ppm). The aromatic proton at δ_H 8.30 ppm is correlated through three bonds with its equal aromatic carbon (δ_C 123.2 ppm); oxyaryl quaternary carbon (δC 150.6 ppm); and quaternary aromatic carbon (δ_C 136.3 ppm). The correlation indicates that the carbonyl group of the carboxylic acid is directly attached to the aromatic ring and is para-substituted with the hydroxyl group. The proton hydroxyl signal did not appear on the spectral because the authentic compound was measured with the CD_3OD solvent. The 1D and 2D NMR spectrum data for compound 1 and the spectrum ^{13}C NMR of 4-hydroxybenzoic acid (1*) from reference Cho et al. (1998) were listed in Table 2.

According to spectral analysis of ^{1}H-NMR, ^{13}C-NMR,

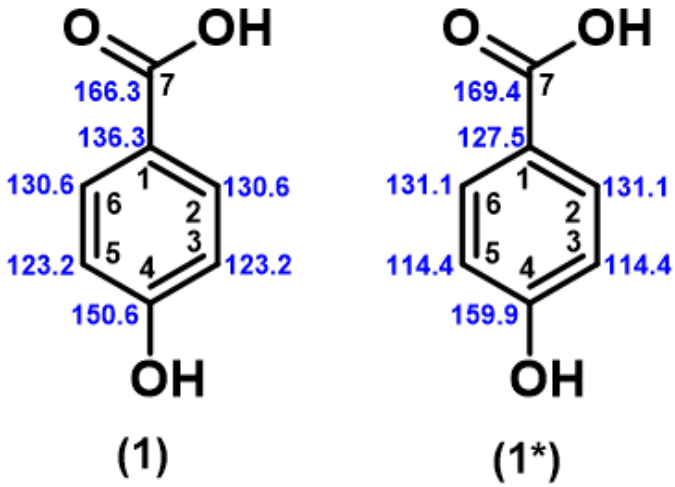

Figure 6. The Placement of the δ_C at the 4-hydroxybenzoic Acid Isolated from Endophytic Fungi *Trichoderma asperellum* (1) and 4-hydroxybenzoic Acid from Reference (1*)

HMQC, and HMBC, it can be described that compound 1 has a para-substituted benzene ring by a hydroxyl group and a carboxylic acid carbonyl. Based on spectrum analysis and after comparing with reference, it was suggested that the chemical structure of compound 1 is 4-hydroxybenzoic acid as shown in Figure 6.

The compound produced by *T. asperellum* from sungkai leaves (Figure 6) showed strong antibacterial and antioxidant activity (MIC ≤ 64 μg/mL; IC50 < 100 μg/mL). Studies have found that the hydroxyl group at a particular position on the aromatic ring enhances the antibacterial effect. At least, one hydroxyl group on ring A (especially at C-7) is essential for the antibacterial activity of flavonoids (Górniak et al., 2019; Sarbu et al., 2019). However, several studies also revealed that a number of hydroxyl groups on two aromatic rings can reduce the antibacterial effect (Farhadi et al., 2019; Shamsudin et al., 2022). This finding strengthens the hypothesis that the special location of the hydroxyl group likely affects the antibacterial activity. Likewise with the antioxidant mechanism due to the presence of aromatic hydroxyl groups.

This antioxidant property is closely associated to the chemical structure of the compound, namely the number of hydroxyl groups, the reciprocal area of para in the aromatic ring, and the grade of esterification. Studies explain that the removal of hydroxyl groups can reduce coplanarity which can reduce the ability of compounds to stroll free radicals (Kubiak Tomaszewska et al., 2022; Platzer et al., 2022). Substitution of the hydroxyl group at its position (C3) with a methyl or glycosyl group can eliminate the antioxidant activity of quercetin (Ferraz et al., 2020; Mucha et al., 2021). Phenolic acids consisting the same number of hydroxyl groups bonded to the aromatic ring do not differ significantly in their antioxidant properties (Spiegel et al., 2020). This indicates that the location of the hydroxyl group significantly affects the antioxidant properties of a compound.

In this study, the compound 4-hydroxybenzoic acid has a double band in the hydroxyl group, causing this compound to be active as an antioxidant.

4. CONCLUSION

The bioactive compound found from *Trichoderma asperellum* isolated from sungkai leaves was 4-hydroxybenzoic acid. This compound has antioxidant and antibacterial activity in the strong category. Based on research, this compound can be used as a new material for drugs with several modifications.

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