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Antioxidant activity of endophytic fungi culture extracts of Christ's thorn jujube (*Ziziphus spina-christi*)

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ABSTRACT: This study aims to explore 50 extracts from fungal endophytes from roots, stems, and leaves of *Z. spina-christi* based on the chemical content and their antioxidant activity. Isolation of fungal endophytes was carried out in CMMA and PDA media. Cultivation was in PDB and GYP media. Determination of antioxidant activity qualitatively by TLC-bioautography and quantitatively by DPPH method. Phytochemical screening of these extracts revealed the presence of alkaloids, phenolics, flavonoids, and tannins. The results showed that seven types of fungal endophytes had antioxidant activity, namely *Trichoderma* sp., *Aspergillus sect. Flavi, Aspergillus sect. Nigri, Aspergillus sect. Terrei, Phomopsis* sp., Sordariomycetes, and Hyphomycetes. One root isolate from PDB (*Aspergillus sect. Terrei* Bi. Ak. 3.3) possesses very strong antioxidant activity (IC_{50} of 9.17 µg/mL and AAI = 3.35) and one stem isolate (*Aspergillus nigri* Bi. Bt. 1.2.2) has strong antioxidant activity (IC_{50} 22.13 µg/mL and AAI = 1.38). The fungal endophyte culture extract from GYP media produced one root isolate (*Aspergillus terrei* Bi. Ak. 3.3) with strong antioxidant activity (IC_{50} 19.54 µg/mL and AAI = 1.57). It can be concluded that the fungal endophytes of *Z. spina-christi* have the potential to be developed as an antioxidant.

KEYWORDS: *Ziziphus spina-christi* Lam. Desf.; Christ's thorn jujube; fungal endophytes; antioxidant.

1. INTRODUCTION

Degenerative diseases are one of the biggest causes of death in the world. According to the World Health Organization (WHO) (2021), 41 million people die from degenerative diseases. Degenerative diseases are caused by decreased organ function, for example, cancer, stroke, coronary heart disease, and diabetes mellitus. Indonesia Basic Health Research data in 2018 showed that the prevalence of cancer in Indonesia increased from 1.4 per 1000 population in 2013 to 1.8 per 1000 population in 2018. Meanwhile, the prevalence of stroke from 7 per 1000 population in 2013 to 10.9 per 1000 population in 2018. The prevalence of heart and diabetes mellitus was 1.5 per 100 population in 2018 [1]. These diseases can be caused by free radicals originating from environmental activities such as air pollutants, radiation, carcinogenic chemicals, cigarette smoke, bacteria, viruses, and the effects of anesthetic drugs and pesticides [2].

Z. spina christi has been used to treat serpent bites, bronchitis, cough, and tuberculosis with the compounds of cyclopeptide alkaloid and triterpenoidal saponins [3]. Traditional medicine as an antioxidant in reducing free radicals is considered an alternative for maintaining health. More researchers are interested in developing antioxidants derived from natural ingredients every year. The methanol extract of Christ's thorn jujube (*Ziziphus spina-christi* Lam. Desf.) has an IC_{50} of 39.1 µg/mL [4], while the methanol extract of the bark has an IC_{50} 51.57 µg/mL [5]. In the ethanolic extract of *Z. spina-christi*, the antioxidant activity was strongly correlated with polyphenolic compounds, with a total polyphenol of 60.47 mg GAE/g dry weight [6]. The methanol extract of *Z. spina-christi* leaves produced an anti-inflammatory activity of 95.3% [7]. Extracts of methanol, ethanol, and ethyl acetate of *Z. spina-christi* stem bark also have antimicrobial activity against *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* [8].

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Pharmacologically, efficacious metabolites are produced by plants, and microorganisms grow in plant tissues, commonly called endophytes. The ability of endophytic microbes, especially fungal endophytes, to induce host plants to produce secondary metabolites is an opportunity for large-scale production of metabolites in a short time without causing environmental damage [9].

There has been no report on endophytic microbes, especially fungal endophytes, that associated with *Z. spina-christi* grows in Indonesia. Therefore, it is very important to conduct research related to the antioxidant activity of *Z. spina-christi* culture extracts and explore their potential to produce active compounds, especially antioxidants. Therefore, this study aims to determine fungal endophytes from *Z. spina-christi* that have the potential as antioxidant compounds and test their antioxidant activity.

2. RESULTS

2.1. Sterilization and isolation of endophytic fungi from *Z. spina-christi*

Endophytic fungi were isolated from the roots, stems, and leaves of the *Z. spina-christi* using the surface sterilization method and placed on Corn Meal Malt Agar (CMMA). The endophytic fungus from the root and stem grew on day 5, while the endophytic from the leaves grew on days 6, 7, 9, and 21. The emerging fungal colonies were purified on Potato Dextrose Agar (PDA) media. Finally, 25 pure isolates of the endophytic fungus were obtained (9 from the root, 11 from the stem, and 5 from the leaf). The same species in a different part of the plant, i.e. *Aspergillus sect. Nigri* (Bi. Ak. 2.1, Bi Ak. 2.2.1 and Bi Bt 1.1) but they had different TLC profiles and antioxidant activity.

2.2. Identification of *Z. spina-christi* fungal endophyte

Table 1 shows that from total of 25 strains of endophytic fungi inhabiting *Z. spina-christi* based on morphological characteristics could be identified into several groups of fungi from the genera *Trichoderma* sp., *Aspergillus sect. Nigri, Aspergillus sect. Flavi, Aspergillus sect. Terrei*, *Penicillium purpurogenum,* Coelomycetes, *Phomopsis* sp., Sordariomycetes, and Hyphomycetes. Several groups of these genera have biological activities such as antifungal [10, antimicrobial [11], anticancer [12], and antioxidant. A group of fungi from *Trichoderma* sp. isolated from mangroves and *Aspergillus sect. Terrei* has excellent potential as a source of antioxidants [13,14]. Further identification based on molecular approach for the most potential strain i.e., *Aspergillus sect. Terrei* strain Bi Ak 3.3 (Figure 1), revealed that the strain based on ITS rDNA sequences has the highest similarity with the *Aspergillus terreus* (Table 2). The phylogenetic analysis of neighbor-joining (NJ) tree showed that the sequence of fungal strain Bi Ak 3.3 isolated from the root of *Z. spina-christi* nested in the *Aspergillus sect. Terrei* clade with the closest sister taxa such as *Aspergillus terreus* strain ATCC 12238 (JQ070071), *A. terreus* strain FJAT-31011 (MH047280), and *A. terreus* strain DTO 403-C9 (MT316343) with bootstrap value 100% (Figure 2).

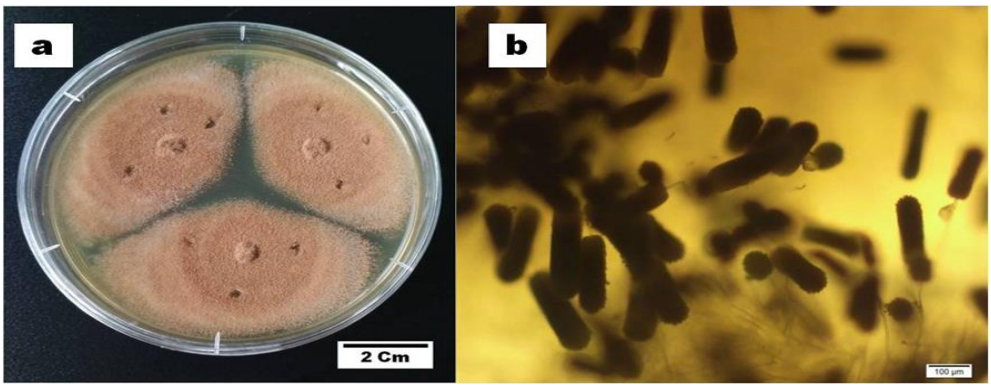

Figure 1. The macroscopic (a) and microscopic view (b) of fungal endophytes with the most potent antioxidant activity *Aspergillus section Terrei* strain Bi.Ak. 3.3 cultures were grown in vitro on artificial media potato dextrose agar (PDA), 7 days incubation at T 27 °C.

Table 1. Taxa of endophytic fungal isolates from *Z. spina-christi* from PDB and GYP media and extract weight

No.	Isolate Code	Part of plant	Taxa	Weight (mg) isolated from PDB*	Weight (mg) isolated from GYP*
1	Bi. Ak. 1.1	root	*Trichoderma* sp.1	91.1	41.1
2	Bi. Ak. 1.2	root	*Trichoderma* sp.2	90.8	72
3	Bi. Ak. 1.3	root	*Trichoderma* sp.3	70.4	41.6
4	Bi. Ak. 2.1	root	*Aspergillus sect. Nigri*-1	118.4	19
5	Bi. Ak. 2.2.1	root	*Aspergillus sect. Flavi*-1	63.9	32.1
6	Bi. Ak. 2.2.2	root	*Aspergillus sect. Nigri*-2	83.3	31.8
7	Bi. Ak. 3.1	root	*Penicillium cf. Purpurogenum*	70.8	49.6
8	Bi. Ak. 3.2	root	Coelomycetes-1	6.3	23.1
9	Bi. Ak. 3.3	root	*Aspergillus sect. Terrei*	239.7	81.8
10	Bi. Bt. 1.1	stem	*Aspergillus sect.Nigri*-3	1474.2	64.8
11	Bi. Bt. 1.2.1	stem	*Phomopsis* sp.-1	52	47.8
12	Bi. Bt. 1.2.2	stem	*Aspergillus sect. Nigri*-3	410.3	57.3
13	Bi. Bt.1.3.1a	stem	*Phomopsis* sp.-1	60.2	27.3
14	Bi. Bt.1.3.1b	stem	Coelomycetes-2	21.5	28.5
15	Bi. Bt. 1.3.2	stem	*Aspergillus sect. Nigri*-3	69.3	50.3
16	Bi. Bt. 2.1	stem	*Phomopsis* sp.-2	20	20
17	Bi. Bt. 3.1	stem	*Phomopsis* sp.-3	24.8	89
18	Bi. Bt. 3.2	stem	*Phomopsis* sp.-4	71.2	31.1
19	Bi. Bt. 3.3	stem	*Phomopsis* sp.-2	26	18.2
20	Bi. Bt. 3.4	stem	*Aspergillus sect. Flavi*-2	460.3	156.2
21	Bi. Da. 1.1	leaf	*Phomopsis* sp.-5	49.2	45.2
22	Bi. Da. 1.2	leaf	*Phomopsis* sp.-6	25.9	19.4
23	Bi. Da. 1.3	leaf	*Phomopsis* sp.-6	31.9	32.2
24	Bi. Da. 2.1	leaf	Sordariomycetes	14.6	38.4
25	Bi. Da. 3.1	leaf	Hyphomycetes	13.3	9.8

*extracts produced by 200 mL endophytic fungi cultured in media (PDB, and GYP).

Table 2. The BLAST result of fungal endophyte with the most potent antioxidant activity *Aspergillus section Terrei* strain Bi.Ak. 3.3 based on ITS rDNA sequence according to NCBI BLAST (https://blast.ncbi.nlm.nih.gov/)

Fungal strain	1st and 2nd closest taxa on NCBI BLAST (https://blast.ncbi.nlm.nih.gov/)
BiAk 3.3	*Aspergillus terreus* strain FJAT-31011 (Accession no: MH047280) [Similarity: 98.37%; Max score: 1072; Total score: 1072; Query coverage: 99%; E-value: 0.0; Max identities: 604/614 (98%); Gaps: 7/614 (1%)]
	Aspergillus terreus strain DTO 403-C9 (Accession no: MT316343) [Similarity: 98.21%; Max score: 1068; Total score: 1068; Query coverage: 99%; E-value: 0.0; Max identities: 604/615 (98%); Gaps: 8/615 (1%)]

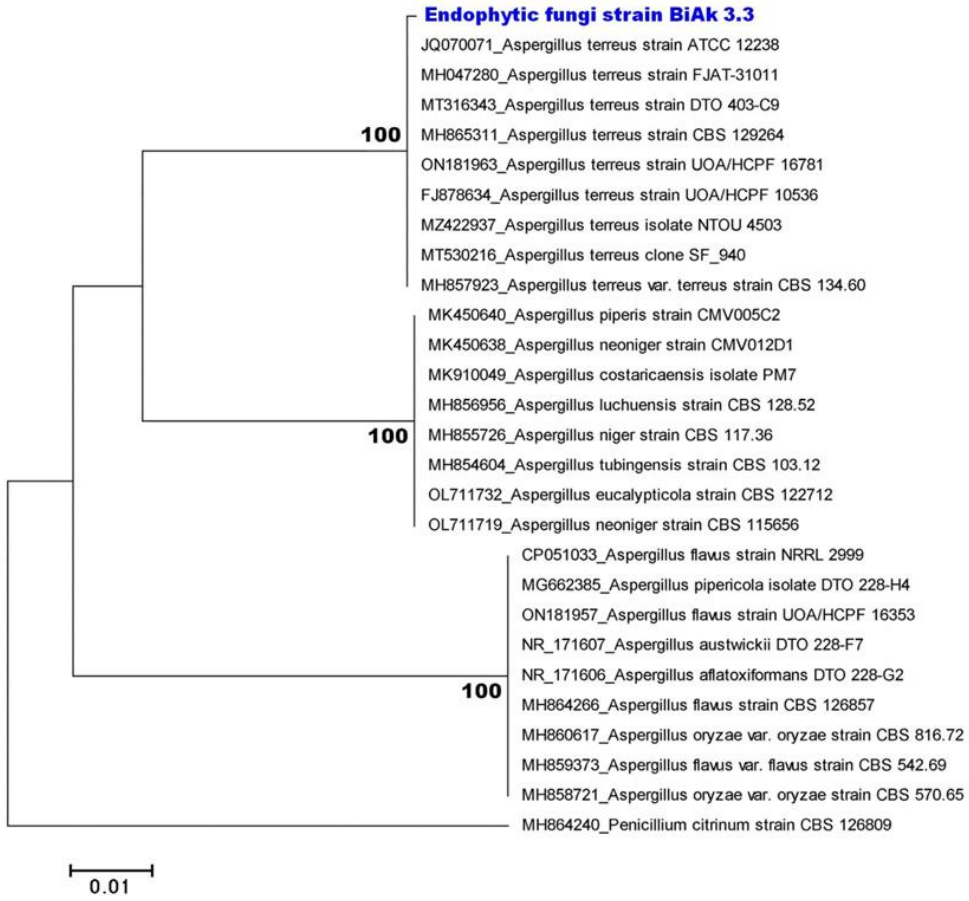

Figure 2. Neighbor-joining tree of fungal endophyte strain BiAk 3.3 based on ITS rDNA sequence and *Penicillium citrinum* as outgroup. Bootstrap values above 75% are recorded at the nodes (1000 replicates).

2.3. Cultivation of *Z. spina-christi* fungal endophyte

Pure isolates of endophytic fungi were cultivated using Potato Dextrose Broth (PDB) and Glucose-Yeast-Peptone Extract (GYP) media. During cultivation, the color of the PDB media was changed, as seen in samples of unidentified Coelomycetes Bi. Ak. 3.2 and Sordariomycetes Bi. Da. 2.1, from clear yellow to cloudy yellow. The color change was caused by fungal activity in utilizing PDB and GYP media nutrients. The difference in color or turbidity in the liquid substrate was caused by the addition of cell mass and the metabolic processes of endophytic fungi in utilizing the nutrients contained in the media [15]. It indicates the production of metabolites in PDB and GYP cultivation media. There are differences in TLC analysis of endophytic fungi extracts from PDB and GYP media and antioxidant activity.

2.4. Extraction of *Z. spina-christi* fungal endophyte

This study used liquid-liquid extraction with ethyl acetate solvent to extract chemical compounds from endophytic fungal isolates. The isolate was mashed to break down the cells so that it could facilitate extraction. Cultivation results were put into a separating funnel. Separation of two phases, namely the aqueous and solvent phases, wherein the solvent phase is the metabolite of the endophytic fungus. The solvent phase was generally in the top layer. This layer was taken because it was a supernatant containing secondary metabolites. The total extracts were 50 isolate extracts (18 from the root, 22 from the stem, and 10 from the leaf) from 25 PDB and 25 GYP media. Fifty extracts were used for compound analysis and antioxidant activity tests. The total weight of each extract is presented in Table 1.

2.5. Chemical compound analysis of *Z. spina-christi* fungal endophyte

Compound separation was performed to analyze the class of chemical compounds from fungal endophyte extract using Thin Layer Chromatography (TLC). The chromatogram profiles were detected under 254 nm and 366 nm UV light, followed by cerium and vanillin reagents spraying. Spot detection of

chemical compounds from endophytic fungal extracts cultured in PDB and GYP media at UV light 254 nm showed the spots muffled fluorescence. The plate emitted a green. At 366 nm, UV light emits purple spots, and the plate reduces fluorescence.

Cerium sulfate reagent was a spot detector for terpenoid, steroid sapogenin, and alkaloid. In contrast, the vanillin reagent (sulfuric acid and vanillin) was a spot detector that can be used to visualize the components of volatile compounds, steroids, phenol, and terpenoid. The PDB and GYP extracts of fungal endophyte cultures showed spots with blue to purple color, which are terpenoid compounds (Figures 3 and 4).

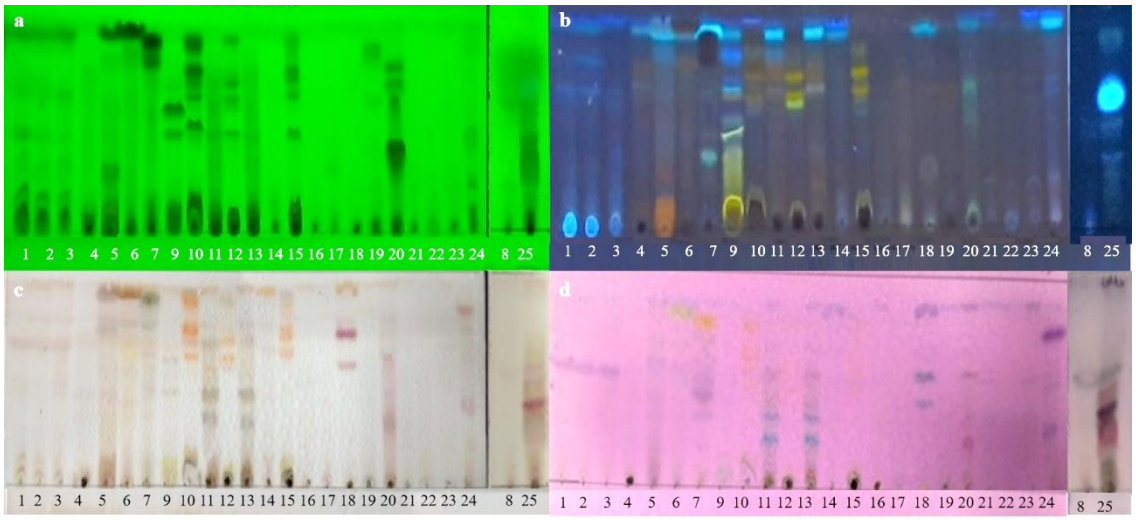

Figure 3. Chromatogram of fungal endophytes extract of *Z. spina-christi* in PDB media, The chromatogram was observed under the following conditions: (a) UV 254 nm, (b) UV 366 nm, after spraying with (c) 1% $Ce(SO_4)_2$, and (d) 1% vanillin in sulfuric acid. Extracts no. 1–25 refer to Table 1. Solvent development is the mixture of dichloromethane – methanol (10:1).

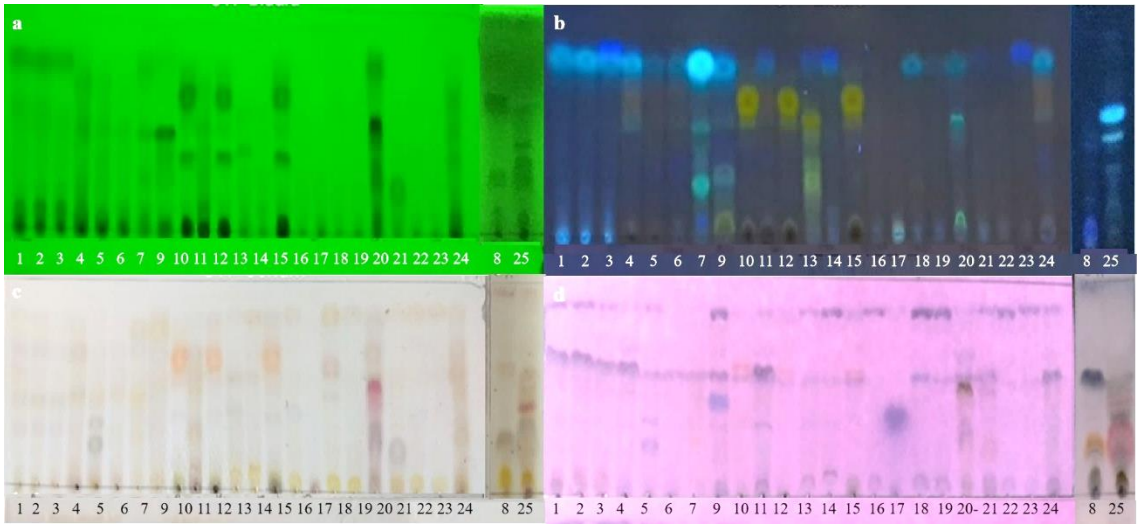

Figure 4. Chromatogram of fungal endophytes culture extract of *Z. spina-christi* in GYP media. The chromatogram was observed under the following conditions: (a) UV 254 nm, (b) UV 366 nm, after spraying with (c) 1% $Ce(SO_4)_2$, and (d) 1% vanillin in sulfuric acid. Extracts no. 1–25 refer to Table 1. Solvent development is the mixture of dichloromethane – methanol (10:1).

2.6. Phytochemical screening of *Z. spina-christi* fungal endophytes

The secondary metabolites of fungal endophyte extracts of *Z. spina-christi* cultivated in PDB and GYP media were determined, and they contained phenolics, flavonoids, and tannins (Table 3). The qualitative phytochemical analysis indicates that saponins were absent in the fungal endophyte culture extracts. The TLC profile confirmed alkaloids' presence in *Z. spina-christi* fungal endophytes extracts (Figure 5), indicated

by the orange-brown spots after the Dragendroff reagent spraying. Endophytic fungi no. 11 and 13 have the same TLC profiles, but they have different AAI with the same category of antioxidant activity (Figure 3)

Table 3. Phytochemical screening of *Z. spina-christi* fungal endophytes from PDB and GYP media.

No.	Taxa	Compounds							
		Phenolics		Flavonoids		Tannins		Saponins	
		PDB	GYP	PDB	GYP	PDB	GYP	PDB	GYP
1	*Trichoderma* sp.	-	-	-	-	-	-	-	-
2	*Trichoderma* sp.	-	-	-	-	-	-	-	-
3	*Trichoderma* sp.	-	-	-	-	-	-	-	-
4	*Aspergillus sect. Nigri*-1	+	+	-	-	-	-	-	-
5	*Aspergillus sect. Flavi*-1	+	+	-	-	-	-	-	-
6	*Aspergillus sect. Nigri*-2	+	+	-	-	-	-	-	-
7	*Penicillium cf. Purpurogenum*	+	+	-	-	-	-	-	-
8	Coelomycetes-1	+	+	+	+	-	-	-	-
9	*Aspergillus sect. Terrei*	+	+	-	-	+	+	-	-
10	*Aspergillus sect. Nigri*-3	+	+	-	-	+	+	-	-
11	*Phomopsis* sp.-1	+	+	-	-	-	-	-	-
12	*Aspergillus sect. Nigri*-3	+	+	-	-	+	+	-	-
13	*Phomopsis* sp.-1	+	+	-	-	-	-	-	-
14	Coelomycetes-2	-	-	-	-	-	-	-	-
15	*Aspergillus sect. Nigri*-3	+	+	-	-	+	+	-	-
16	*Phomopsis* sp.-2	-	-	-	-	-	-	-	-
17	*Phomopsis* sp.-3	-	-	-	-	-	-	-	-
18	*Phomopsis* sp.-4	+	+	-	-	-	-	-	-
19	*Phomopsis* sp.-2	-	-	-	-	-	-	-	-
20	*Aspergillus sect. Flavi*-2	+	+	+	+	-	-	-	-
21	*Phomopsis* sp.-5	+	+	+	+	-	-	-	-
22	*Phomopsis* sp.-6	-	-	-	-	-	-	-	-
23	*Phomopsis* sp.-6	-	-	-	-	-	-	-	-
24	Sordariomycetes	+	+	-	-	-	-	-	-
25	Hyphomycetes	+	+	-	-	-	-	-	-

(+) : containing the compound, (-): not containing the compound.

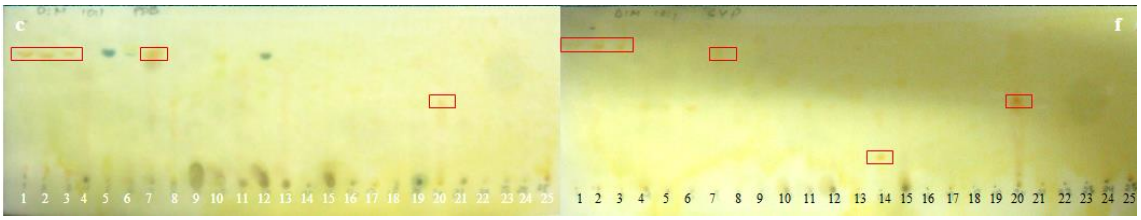

Figure 5. Chromatogram of fungal endophytes culture extract of *Z. spina-christi* in PDB (c) and GYP (f). The chromatogram was observed after spraying with Dragendroff reagent. Extracts no. 1–25 refer to Table 1. The eluent system was dichloromethane – methanol (10:1).

2.7. Measurement of antioxidants by TLC-bioautography

TLC-bioautography was chosen as a qualitative antioxidant test to determine the antioxidant activity of the fungal endophytes culture extract as indicated by the color change on the plate after being sprayed with 2,2-diphenyl-1-picrylhydrazil (DPPH). The results showed that 13 extracts from PDB media and 9 extracts from GYP media gave positive results of antioxidant activity marked by a yellowish zone in the spot area on the silica gel plate after being sprayed with DPPH (data not shown). The yellowish zone is formed when the antioxidant compounds in the sample of endophytic fungal culture extract donate one atom /molecule or hydrogen atom to the DPPH nitrogen radical, resulting in a color change from purple to yellow [16].

Twenty-two extracts were spotted again on a new silica gel plate and developed with a mixture of dichloromethane - methanol (10:1). The results showed absorption between the samples on the silica gel plate in 22 eluted fungal endophytes culture extract extracts. After spraying with DPPH 0.2%, the yellow zone indicated that certain spots could act as antioxidants (Figures 6 and 7).

Figure 6. Bio-autogram of antioxidant activity of fungal endophyte culture extracts of *Z. spina-christi* in PDB media developed with dichloromethane: methanol (10:1) observed (a) 254 nm, (b) 366 nm, and (c) 30 min after sprayed by 0.2% DPPH solution in methanol. Extracts no. 1–25 refer to Table 1.

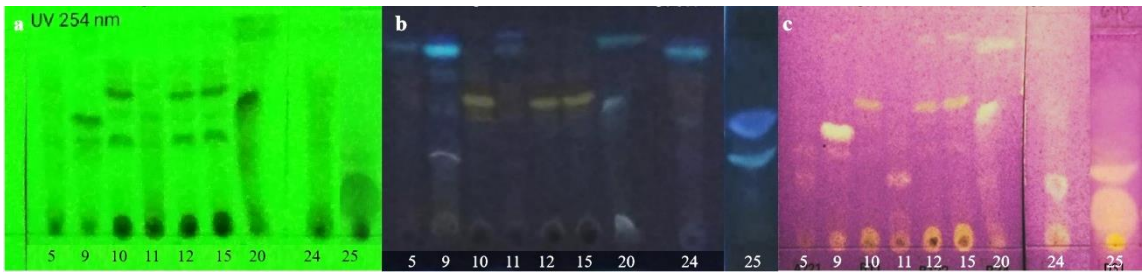

Figure 7. Bio-autogram of antioxidant activity of fungal endophyte culture extracts of *Z. spina-christi* in GYP media developed with dichloromethane: methanol (10:1) observed (a) 254 nm, (b) 366 nm, and (c) 30 min after sprayed by 0.2% DPPH solution in methanol. Extracts no. 1–25 refer to Table 1.

2.8. Measurement of antioxidants using the DPPH method

Free radical attenuation is expressed by %, and the IC_{50} value (µg/mL) expresses antioxidant activity, which indicates the antioxidant concentration required to produce 50% DPPH radical inhibition. The smaller the IC_{50} value, the greater the antioxidant activity [17]. The antioxidant test results of 22 fungal endophytes culture extracts from the roots, stems, and leaves of *Z. spina-christi* can be seen in Table 4. On PDB media, one root isolate (*Aspergillus sect. Terrei*) had very strong antioxidant activity [IC_{50} 9.17 µg/mL and Antioxidant Activity Index (AAI) = 3.35] and one stem isolate (*Aspergillus sect. Nigri*) had strong antioxidant activity (IC_{50} 22.13 µg/mL and AAI = 1.38). In GYP media, one root isolate (*Aspergillus sect. Terrei*) had strong antioxidant activity (IC_{50} 19.54 µg/mL and AAI = 1.57). The presence of phenolics and tannins was discovered through the phytochemical screening in *Aspergillus sect. Terrei*. (+)-Catechin was used as a positive control with an AAI value of 11.17 (very strong category).

3. DISCUSSION

Based on the morphological examination, three fungi isolates obtained from roots (isolates no. 1, 2, and 3) are members of the genus Trichoderma. Comparing the TLC chromatogram patterns of detected compounds between these fungal endophyte extracts revealed that the results were similar, with even the same Rf (retention factor) values and color. Isolates with identical morphological characteristics and TLC

chromatogram patterns indicate that the isolates produce the same secondary metabolites [18]. As a result, it could be assumed that these fungal endophyte extracts (isolates no. 1, 2, and 3) have similar secondary metabolites and must be from the same species. Members of Trichoderma can be found in a variety of environments and reside as endophytes in plant tissues. There are currently around 340 species classified in the genus of *Trichoderma* [19], and their species can produce a broad spectrum of antibiotics and parasitize other fungi [20].

Table 4. AAI category of fungal endophyte culture extracts of *Z. spina-christi*.

No.	Taxa	IC50 (µg/mL)		AAI		Antioxidant category	
		PDB	GYP	PDB	GYP	PDB	GYP
1	*Trichoderma* sp.	> 128	NT	-	NT	Weak (+)	-
2	*Trichoderma* sp.	> 128	NT	-	NT	Weak (+)	-
3	*Trichoderma* sp.	> 128	NT	-	NT	Weak (+)	-
4	*Aspergillus sect. Nigri*-1	NT	NT	NT	NT	-	-
5	*Aspergillus sect. Flavi*-1	> 128	> 128	-	-	Weak (+)	Weak (+)
6	*Aspergillus sect. Nigri*-2	> 128	NT	NT	NT	Weak (+)	-
7	*Penicillium cf. Purpurogenum*	NT	NT	NT	NT	-	-
8	Coelomycetes-1	NT	NT	NT	NT	-	-
9	*Aspergillus sect. Terrei*	9.17	19.54	3.35	1.57	Very strong (++++)	Strong (+++)
10	*Aspergillus sect. Nigri*-3	47.56	106.18	0.64	0.28	Moderate (++)	Weak (+)
11	*Phomopsis* sp.-1	115.29	> 128	0.26	-	Weak(+)	Weak (+)
12	*Aspergillus sect. Nigri*-3	22.13	40.44	1.38	0.76	Strong(+++)	Moderate (++)
13	*Phomopsis* sp.-1	82.92	NT	0.37	NT	Weak(+)	-
14	Coelomycetes-2	NT	NT	NT	NT	-	-
15	*Aspergillus sect. Nigri*-3	46.01	38.62	0.66	0.79	Moderate (++)	Moderate (++)
16	*Phomopsis* sp.-2	NT	NT	NT	NT	-	-
17	*Phomopsis* sp.-3	NT	NT	NT	NT	-	-
18	*Phomopsis* sp.-4	NT	NT	NT	NT	-	-
19	*Phomopsis* sp.-2	NT	NT	NT	NT	-	-
20	*Aspergillus sect. Flavi*-2	> 128	79.70	-	0.38	Weak(+)	Weak (+)
21	*Phomopsis* sp.-5	NT	NT	NT	NT	-	-
22	*Phomopsis* sp.-6	NT	NT	NT	NT	-	-
23	*Phomopsis* sp.-6	NT	NT	NT	NT	-	Weak (+)
24	Sordariomycetes	NT	> 128	NT	-	-	Weak (+)
25	Hyphomycetes	> 128	> 128	-	-	Weak(+)	-
26	(+)-Catechin	2.751		11.17		Very strong (++++)	

*NT: Not tested, did not show qualitative antioxidant activity.

Furthermore, two fungal isolates obtained from roots (isolates no. 4 and 6) and three isolates from stems (isolates no. 10, 12, and 15) have similar morphological characteristics. These fungal isolates belong to the *Aspergillus section Nigri*. The TLC chromatogram patterns of these fungal endophyte extracts differ despite their similar morphology. They form three distinct patterns, indicating that they are from three different species. Isolates obtained from the root (isolates 4 and 6) have different TLC chromatogram patterns, from one another, and for this reason, isolates no.4, and 5 were identified as *Aspergillus sect. Nigri*-1, and *Aspergillus sect. Nigri*-2, respectively. Three isolates obtained from the stem (isolates no. 10, 12, and 15) have identical TLC chromatogram patterns, and are classified as *Aspergillus sect. Nigri*-3. The previous study by Ilyas et al. (2008) also showed that the endophytic fungi from the leaves, stems, fruits, and roots of

Uncaria gambier Roxb. (Rubiaceae) which have identical morphological characters, TLC patterns were identified as the same species [18].

As a result, the phytochemical content of endophytic fungal species isolated from different plant parts was comparable. *Aspergillus* is one of the earliest genera of fungi containing 250 species and is divided into nine sections: *Flavi*, *Fumigati*, *Nigri*, *Udagawae*, *Cricumdati*, *Versicolor*, *Usti*, *Terrei*, and *Emericella* [21,22]. *Aspergillus* has produced a variety of valuable metabolites, including antibiotics and the cholesterol-lowering drug [23]. *A. section Nigri*, also known as black aspergilli, has many common species in the environment and has considerable importance because of its ability to produce enzymes and organic acids [24]. There are 26 species in the *A. section Nigri*, including the new species *A. saccharolyticus* [25]. Identifying the *A. section Nigri* at the species level is difficult based solely on the traditional method (phenotypic and morphological characteristics), which frequently fails to distinguish between closely related species [26,27].
In addition to the *Aspergillus section Nigri*, our data show that other *Aspergillus*-derived endophytic fungal isolates are isolates no. 5 and 9 (fungi isolates obtained from root) and isolate no. 20 (fungi isolate obtained from the stem). Isolate no. 9 is an *Aspergillus section Terrei* member, while the other isolates belong to members of *Aspergillus section Flavi*. Despite their similar morphology, isolates no. 5 and 20 have distinct chromatogram patterns, implying that these isolates produce different secondary metabolites. As a result, isolates no. 5 and 20 were identified as *Aspergillus sect. Flavi*-1, and *Aspergillus sect. Flavi*-2, respectively. *Aspergillus section Flavi* comprises 34 different species that can be divided into eight distinct clades [28,29]. Aflatoxin B1 is the most toxic of the many naturally occurring secondary metabolites produced by fungi, produced by several species of the section *Flavi* [30]. The taxonomy of this fungus group is still highly complex; species classification may be difficult due to the extensive divergence of morphological characters caused by a high level of genetic variability. According to recent data, several species assigned to section *Flavi* cannot be distinguished solely by morphological features, necessitating a molecular strategy involving real-time PCR, RAPD, and small digestion of nuclear DNA [30].

Morphological analysis revealed two fungal isolates obtained from the root (isolate no. 8), and the stem (isolate no. 14) are Coelomycetes. Despite their similar morphology, the TLC chromatogram patterns of these fungal endophytes differ. Coelomycetes comprise approximately 1000 genera and 7000 species that can be endophytic, pathogenic, or saprobic [31]. As a result, both fungi isolates (isolates no. 8 and 14) indicate that they are from different genera or species levels, and are classified as Coelomycetes-1, and Coelomycetes-2, respectively. Endophytic fungi of the class Coelomycetes can also produce metabolites with interesting biological activities. Wulansari et. al. reported unidentified Coelomycetes isolated from the plant *Archangelesia flava* that produce a strong antifungal metabolite pachybasin.[32].

Phomopsis sp. is the most commonly isolated fungus in this study, i.e., nine out of twenty-five isolates were obtained. Six out of nine isolates are from the stem (isolates no. 11, 13, 16, 17, 18, and 19), and three are isolated from the leaf (isolates no. 21, 22, and 23). Comparing the chromatogram patterns of endophytic fungal extracts on TLC revealed six distinct patterns. It indicated that they belong to six different species. The fungi isolates no. 17, 18, and 21 show their own specific TLC patterns. The remaining six isolates show three distinct patterns, and two isolates show identical TLC chromatogram patterns. Isolates 11 and 13 have similar profiles, as isolates 16 and 19, and isolates 22 and 23 also have similar profiles. As a result, isolates no 11 and 13 were classified as *Phomopsis* sp.-1, isolates 16 and 19 as *Phomopsis* sp.-2, isolates 22 and 23 as *Phomopsis* sp.-6, and the other three isolates (isolates no.17, 18, and 21) were identified as *Phomopsis* sp.-3, *Phomopsis* sp.-4, and *Phomopsis* sp.-5, respectively. Endophytic fungi have been isolated from the root of *Uncaria gambier* Roxb. (Rubiaceae) from West Sumatera [18]. Morphological observations (microscopic and macroscopic) of two isolates of endophytic fungi isolated from the root of *Uncaria gambier* var nasi reveal two Aspergillus species, indicating that they belong to different species. TLC chromatogram patterns of endophytic fungi extracts show distinct profiles, validating those claims. Two isolates (GNAP-2 and GNAP-5) belong to the *Aspergillus sp*. and *Aspergillus niger*, respectively. *Phomopsis* contains over 900 species from diverse hosts [33]. This genus has long been recognized as a rich source of bioactive secondary metabolites with many structures. Endophytic *Phomopsis* sp. from the twigs of *Salic gracilostyla* var. *melanostachys* produced phomopsicalashin, which has antibacterial and antifungal activity [34], and taxol, which has anticancer activity, was produced by an endophytic *Phomopsis* sp. from the leaves of *Taxus cuspidata* (Japanese yew) [35]. *Phomopsis* sp. BCC 1323 is an endophyte from *Tectona grandis* that produces phomoxantone A and B, xanthone dimers with antimalarial, antitubercular, and cytotoxic properties [36]. Most of these metabolite-producing strains are only recognized at the generic level due to the practical difficulty in identifying *Phomopsis* at the species level [33].

4. CONCLUSION

From the isolation, a total of 25 selected endophytic fungal strains were isolated from roots (9 strains), stems (11 strains) and leaves (5 strains) of *Z. spina-christ*. Morphological identification revealed seven fungal taxa found in roots, namely *Aspergillus section Flavi-1*, *A. sect. Nigri-1*, *A. sect. Nigri-2*, *A. sect. Terrei*, Coelomycetes-1, *Penicillium purpurogenum*, and *Trichoderma* sp.; seven fungal taxa were found in stems, namely *A. sect. Flavi-2, A. sect. Nigri-3*, Coelomycetes-2, and *Phomopsis* sp. 1-4; and four fungal taxa in leaves, namely Hyphomycetes, Sordariomycetes, and *Phomopsis* sp. 5-6. Seven species of fungal endophytes found in *Z. spina-christi* have potential as antioxidants, namely *Trichoderma* sp., *Aspergillus* sect. Flavi, *Aspergillus* sect. Nigri, *Aspergillus* sect. Terrei, *Phomopsis* sp., Sordariomycetes and Hyphomycetes. The presence of alkaloids, phenols, flavonoids and tannins was detected by phytochemical screening of these extracts. On PDB media, a root isolate had very strong antioxidant activity (IC_{50} 9.17 µg/mL and AAI = 3.35) and a stem isolate had strong antioxidant activity (IC_{50} 22.13 µg/mL and AAI = 1.38). In GYP media, a root isolate had strong antioxidant activity (IC_{50} 19.54 µg/mL and AAI = 1.57). The results of this study also showed that the differences in growth medium also affected the differences in biological activity, indicating the differences in secondary metabolites.

5. MATERIALS AND METHODS

5.1. Sample and determination

Christ's thorn jujube (*Z. spina-christi* Lam. Desf.) was obtained from the Research Institute for Spices and Medicinal Plants, Bogor. Identification of the plant was carried out at the Herbarium Bogoriense, Research Center for Biology, National Research and Innovation Agency, Cibinong. *Z. spina-christi* plant consisting of leaves, stems, and roots were washed under clean running water. Then, it was sterilized by soaking in 100 mL of 70% ethanol for 1 min, followed by soaking in 100 mL of 5.3% sodium hypochlorite for 5 min and then in 100 mL of 70% ethanol for 30 s. Samples were dried aseptically in laminar airflow, and each section was cut for 1×1 cm^2 [37,38].

The sterilized samples were cultivated on CMMA media, then incubated at 27 °C for 7 days. After incubation, the fungal colonies were purified by taking a small number of hyphae that grew on CMMA media using a sterile ose needle and placing them on PDA media. Furthermore, the PDA media was incubated at 27 °C until pure isolates emerged. Finally, pure isolates grew well on PDA media and were labeled with isolate codes [39].

5.2. Identification of *Z. spina-christi* fungal endophyte

Initial endophytic fungi identification is carried out based on morphological characteristics. Morphological identification was conducted by observing both macroscopic and microscopic properties. Macroscopic characterizations include observations on color, colony shape, surface, texture, exudate drop, and reverse color. Microscopic slides of each selected strain were prepared using lactophenol as a mounting medium. Microscopic characterizations were performed on a light microscope by observing hyphae, hyphae pigmentation, septate, clamp connection, conidia, spores, and other reproductive structures [40].

The representative potential strain then selected for further identification using the molecular approach. The molecular identification conducted by analyzing the DNA sequence of an internal transcribed spacer (ITS1 and ITS2) of rDNA regions, includes the 5.8S rRNA. The total fungal genomic DNA was isolated using Nucleon PhytoPure, plant and fungal DNA extraction kits (GE Healthcare) according to the manufacturer`s instructions. DNA amplification of the ITS rDNA region was performed by polymerase chain reaction (PCR). PCR amplification was performed in 25 µL reaction mixtures containing 10 µL distilled water, 12,5 µL GoTaq Green Master Mix (Promega), 0.5 µL DMSO, 0,5 µL each primer (10 pmol), and 1 µL (5 to 10 ng) extracted genomic DNA as a template. The primer set of ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) was used to amplify approximately 550 nucleotides from ITS1 and ITS 2 including 5.8S rDNA [41]. Amplification was performed in a TaKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.), programmed under following conditions: initial denaturation at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR products were then subjected to purification and sequence analysis.

Initial phylogenetic tree construction of selected strains was conducted by editing the raw sequence data using ChromasPro (http://www.technelysium.com.au/ChromasPro.html). The assembled sequences were aligned with those downloaded from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nlh.gov/) using the Muscle (http://www.ebi.ac.uk/Tools/msa/muscle). The

phylogenetic analyses of sequence data were done based on the neighbor-joining (NJ) method [42] using Molecular Evolutionary Genetics Analysis (MEGA) version 7 program [43]. The reliability of each branch was evaluated by bootstrapping with 1000 resampling.

5.3. Cultivation of *Z. spina-christi* fungal endophyte

The hyphae of pure isolates of *Z. spina-christi* fungal endophytes were taken with an ose needle and cultivated in 25 Erlenmeyer containing 200 mL of GYP liquid media and 25 Erlenmeyer containing 200 mL of PDB media [44]. Furthermore, the Erlenmeyer was incubated for 21 days. After 21 days of incubation, the culture media and the biomass were macerated with ethyl acetate [45].

5.4. Extraction of *Z. spina-christi* fungal endophyte

After incubation, fifty cultures were added with 50 mL of ethyl acetate. Then, the fungal endophyte biomass was taken and blended. Next, put back into Erlenmeyer and homogenized using the magnetic stirrer. Finally, put the endophytic biomass and culture media into a separating funnel while filtering. Extraction was carried out by the liquid-liquid method by adding 250 mL of ethyl acetate solvent into a separating funnel to form 2 phases while shaking vigorously. Phase 1 and phase 2 were separated. Phase 1 contains water and pulp, while phase 2 contains extract dissolved in ethyl acetate. Furthermore, phase 2 was concentrated with a vacuum rotary evaporator until a thick extract was obtained. The concentrated extract was put in a vial and stored at -20 °C [46].

5.5. Chemical compound analysis of *Z. spina-christi* fungal endophyte using TLC

Chemical compound analysis was carried out on a silica gel plate (GF254, Merck). The extract was prepared at a concentration of 10 mg/mL. A total of 10 µL extract was spotted using a capillary tube on a silica gel plate and eluted with dichloromethane - methanol (10:1). Fifty extracts were spotted. The separated chemical compounds were visualized under UV light at 254 nm and 366 nm, followed by spraying 1% $Ce(SO_4)_2$ and 1% vanillin in sulfuric acid [37].

5.6. Phytochemical screening of *Z. spina-christi* fungal endophyte

The fungal endophyte extracts were analyzed for their chemical compounds using the standard procedure to identify alkaloids [46], phenolics [47], flavonoids [48], saponins [47], and tannins [48].

5.7. Antioxidant measurements using TLC-bioautography

The extract was prepared at a concentration of 10 mg/mL. Ten µL of extracts and (+)-catechin were spotted using a capillary tube on a TLC plate. Fifty extracts were spotted. The TLC plates were dried and sprayed with 0.2% DPPH in methanol. Incubation was carried out for 30 min in a dark room. Yellow spots on the purple background indicated antioxidant activity. Furthermore, 22 active extracts were re-spotted using a capillary tube with as much as 10 µL of the extract on the plate and eluted with dichloromethane - methanol (10:1). After drying, the TLC plates were visualized under UV light at 254 nm and 366 nm, followed by spraying 0.2% DPPH in methanol. Incubation was carried out for 30 min in a dark room. Spots producing a yellow zone on a purple background indicated antioxidant activity [37].

5.8. Antioxidant measurements using the DPPH method

Antioxidant activity was measured by using the DPPH method [37]. (i) DPPH solution was prepared at a concentration of 61.5 µg/mL in methanol. (ii) Sample solution and (+)-catechin standard was prepared at a concentration of 10240 µg/mL in DMSO. (iii)One hundred µL of methanol p.a. was added to each 96-well microplate. (iv) 95 µL methanol p.a was put in rows A1-A12. (v) 5 µL sample was put in rows 1 A1-A12. (vi) Serial dilution was made by homogenizing rows 1 A1-A12, then 100 µL was taken and put into row 2 (B1-B12), then 100 µL was taken and put into row 3 (C1-C12). In the last dilution process, 100 µL was taken and discarded. (vii) Steps iv-vi were applied to other samples and (+)-catechin standard in different wells. (viii) All wells were added with 100 µL of DPPH with a 61.50 µg/mL concentration. (ix) Control 100% DPPH filled with 100 µL methanol p.a + 100 µL DPPH. (x) 0% control filled with 200 µL of methanol. (xi) Incubation was carried out for 90 min in a dark room at 22-24 °C and measured with a Varioscan flash at a wavelength of 517 nm. The antioxidant activity capacity was determined based on the IC_{50} and AAI values. The IC_{50} index is calculated as follows:

$$IC\ (\%) = [(\Delta\ DPPH - \Delta\ Sample) / (\Delta\ DPPH)] \times 100\% \quad \text{Eq. 1}$$

where:

Δ DPPH = Absorbance of (DPPH 100% - DPPH 0%)
Δ Sample = Absorbance of (sample - DPPH 0%)

The calculation of the Antioxidant Activity Index (AAI) value is as follows:

AAI = The final concentration of DPPH in the reaction (µg/mL) / IC_{50} (µg/mL) Eq. 2

The classification of AAI was very strong (AAI > 2), strong (AAI between 1-2), moderate (AAI between 0.5-1), and weak (AAI < 0.5) [49].

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