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Isolation and Identification of Endophytic Fungi Producing of Antioxidant Compound from *Azadirachta indica* A.juss Based on gen 18s rRNA

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ABSTRACT

Endophytic fungi are microbes living in plant tissues and are currently being explored as an alternative to bioactive compounds because they produce metabolites usable as medicinal raw materials. Therefore, this study aims to isolate and identify endophytic fungi that produce antioxidant compounds from neem (*Azadirachta indica* A.juss) leaf. The isolation process produced 4 endophytic fungi coded as FDM 1, FDM 2, FDM 3, and FDM 4. Subsequently, the isolates were fermented for 10 days using PDY media, and then extracted using ethyl acetate solvent in a ratio of 1:1. The TLC method detected the antioxidant compounds in the extract, and FDM 2 was observed to contain antioxidant compounds, which was indicated by yellow spots on the stains after spraying with DPPH. Furthermore, the antioxidant activity test showed that FDM 2 has an IC50 value of 54.93 ppm, while the molecular identification results based on the 18s rRNA gene showed that FDM 2 was similar to Lentinus squorosulus with an identity percentage of 99.55%.

1. INTRODUCTION

Endophytic microbes live in plant tissues and form colonies without harming the host plant [1]. They are found in the flowers, fruits, stems, leaves, roots, and seeds, where they protect the host from environmental stress and microbial competition [2]. Furthermore, these microbes live in a mutually beneficial symbiosis with the host, where they get nutrients from the plant's metabolism, and produce active compounds in the form of secondary metabolites that protect the host from diseases [3]. Endophytic fungi have attracted taxonomists and chemists in recent years because they produce bioactive compounds [4].

[1] in stated that these microbes produce metabolites according to the parent plant, hence, they are used as a source of secondary metabolites, such as alkaloids, flavonoids, phenolics, steroids, and terpenoids [5]. These fungi also produce natural products used as agricultural chemicals, antibiotics, immunosuppressants, antiparasitics, antioxidants, anticancer, antidiabetic, and antifungal agents. One of the endophytic microbes in question is fungus present in medicinal plants, and a potential source of secondary metabolites [6].

The neem plant contains bioactive compounds in its stems, leaves, and seeds, and almost all the parts have medicinal properties. Its leaves contain bioactive compounds, including steroids, phenolics, terpenoids, glycosides, alkaloids, flavonoids, and tannins [7]. Furthermore, the leaf extract has antioxidant activity that delays or slows down the oxidation rate of materials that are easily oxidized by free radicals [8]. Discovered that neem (*Azadirachta indica* A.Juss) leaf grown in India has strong antioxidant activity, while flavonoids from *Azadirachta Indica* have also been reported to have antioxidant activity [9]. Moreover, the leaves, flowers, and bark of the neem tree have strong antioxidant potential [10]. Based on the study background, which states that endophytic fungi produce bioactive compounds with the same efficacy as the host, it is necessary to carry out this isolation and identification of fungi with antioxidant activity from neem leaves.

2. Materials and methods

2.1 Preparation Sample

The sample used was neem (*Azadirachta indica* A.Juss) leaf, which was disinfected with 70% ethanol for 1 minute, 5.20% sodium hypochlorite for 1 minute, and then rinsed with sterile distilled water 3 times. Subsequently, the sample was drained and cut with a sterile scalpel knife with a size of 1 x 1 cm.

2.2 Endophytic Fungus Isolation

Approximately ± 15 mL of PDA medium was evenly placed in a petri dish and allowed to solidify. The sample was then placed on the surface of the medium and incubated at 25ºC for ± 4 x 24 hours. The endophytic fungal isolates obtained were transferred to a petri dish containing a new PDA medium, then incubated at 25ºC for ± 4 x 24 hours, and it was used in the following process.

2.3 Fermentation and Extraction

The fungi isolate was fermented using 200 mL of PDY medium in 500 mL Erlenmeyer, while the fermentation process was carried out using a shaker at a speed of 150 rpm for 10 days. Subsequently, the fermented product was filtered to separate the liquid and biomass. The fermented liquid was then extracted twice using a liquid-liquid extraction method with 200 mL of ethyl acetate (1:1 v/v) solvent.

2.4 Detection of Antioxidant Compounds by TLC Method

Detection of antioxidant compounds from the extract using the TLC method was carried out by spotting the extract on a silica gel plate F254, then eluted with chloroform and methanol eluent in a ratio of 4:1. The stains that appeared were observed on the UV lamps at 366 nm and 256 nm, then sprayed with a DPPH solution, and the yellow spot was measured to obtain the Rf value.

2.5 Measurement of Antioxidant Activity

The stock solution was prepared by dissolving 25 mg of the extract in 25 ml of absolute ethanol, and a solution with a concentration of 1000 ppm was obtained. About 3.95 mg of DPPH was dissolved in 25 ml of absolute ethanol to obtain a 0.4 mM DPPH solution.

75 µl, 150 µl, 225 µl, 300 µl, and 375 µl of the prepared stock solution were pipetted into a volumetric flask, then 900 L of 0.4 mM DPPH was added, and the volume was made up with absolute ethanol until 5 mL. Finally, the mixture was incubated in unlighted room for 30 minutes, and its antioxidant activity was measured at the maximum wavelength using a UV-Vis spectrophotometer.

2.6 Macroscopic and Microscopic Identification

Identification of the fungi was carried out macroscopically and microscopically. The macroscopic

identification was carried out to determine the morphology of the fungal isolates by direct observation of each isolate, while microscopic determined the microscopic characteristics by observing under a microscope with a magnification of 400 times.

2.7 Molecular Analysis

2.7.1 DNA Extraction

DNA extraction was carried out to separate the genome from other molecules in the cell. The process was carried out with the Geneaid PrestoTM Mini gDNA Tissue kit, which is a tissue DNA extraction kit. Furthermore, 25 mg of the fungus's mycelium was taken, cut into small pieces, placed in a 1.5 mL Eppendorf tube, and 180 L of ATL buffer was added to facilitate the lysis process. 20 L of Proteinase K was then added to break the peptide bonds in the mycelia protein. Subsequently, it was incubated at 60°C for 30 minutes, thereby destroying the fungal mycelia. The following stage was lysis, where GBT buffer was added to the sample, and the mixture was incubated for 10 minutes at 60°C to accelerate cell lysis. Absolute ethanol was then added to collect DNA, this stage is also called DNA binding because the DNA samples were collected. Meanwhile, a high ethanol concentration did not damage the DNA, but the higher the concentration of ethanol used, the stronger the ethanol collects DNA. The DNA obtained was then transferred into the GD column, where the matrix on the column bind to it while the contaminants are suspended. Washing was also carried out using a wash buffer to remove contaminants while the DNA remained bonded to the matrix. Subsequently, the DNA rehydration process was carried out to liquefy or release DNA as they are in the form of sediment. Rehydration is then performed by adding a pre-heated Elution buffer solution that dissolve DNA. The DNA products were produced after the rehydration process.

2.7.2 DNA Concentration Measurement

Measurement of DNA concentration was carried out with a nanodrop spectrophotometer. The measurement began with cleaning the pedestal where the sample was placed (pedestal) using ddH2O, and measuring 1 µl of the blank, then clicking measurement on the PC software. After measuring the blank, 1 µl of the DNA sample was measured, and the measurement on the PC software was clicked. Before the engine was turned off, the pedestal was cleaned again using ddH2O.

2.7.3 PCR

PCR identification was carried out with Forward ITS 1 and Reverse ITS 4 primers, which specifically amplify the following targets: Forward Primer: (5' - TCCGTAGGTGAACCTGCGG - 3') Reverse Primer: (5' - TCCTCCGCTTATTGATATGC - 3'). Subsequently, 38 amplifications cycles were performed, namely predenaturation at 94 ^{o}C for 3 minutes, denaturation at 94 ^{o}C for 1 minute, annealing at 52 ^{o}C for 1 minute, extension at 72 ^{o}C for 2.5 minutes and post extension at 72 ^{o}C for 10 minutes.

2.7.4 Sequencing

The DNA amplification results of *Azadirachta indica* A.juss were used as samples in the sequencing process with the services of the company PT. Indonesian Science Genetics. This step was useful for obtaining data on the nucleotide sequence of the target region from the plant sample.

2.8 Phylogenic Analysis

The obtained sequences were analyzed with the alignment contained in the Basic Local Alignment Search Tool (BLAST) on the NCBI portal. Meanwhile, the phylogenetic tree was created using MEGA 6.0 software, then constructed with the Test Neighbor-joining tree, and finally tested with the Bootstrap method.

3. RESULT AND DISCUSSION

3.1 Isolation of Neem (Azadirachta indica A.juss) Leaf Endophytic Fungi

The isolation process of endophytic fungi from neem (*Azadirachta indica* A.juss) leaf was carried out using PDA media. Fresh neem leaf was sterilized with NaClO and ethanol to remove dirt on the sample surface. Furthermore, Endophytic fungi were obtained from the isolation process and were coded as FDM 1, FDM 2, FDM 3, and FDM 4. The results of the isolation are shown in Figure 1.

The findings from the isolation process prove that endophytic fungi are present in neem leaf tissue. This is in line with Carroll and Clay (1988) that endophytic fungi are present in plant tissue such as leaves, flowers, twigs, and plant roots. Meanwhile, the isolates produced from the leaves are pure and free from contamination with other microorganisms as the control's medium, rinse water, and room did not show the growth of microorganisms. This finding is consistent with the modification of surface disinfection by [11].

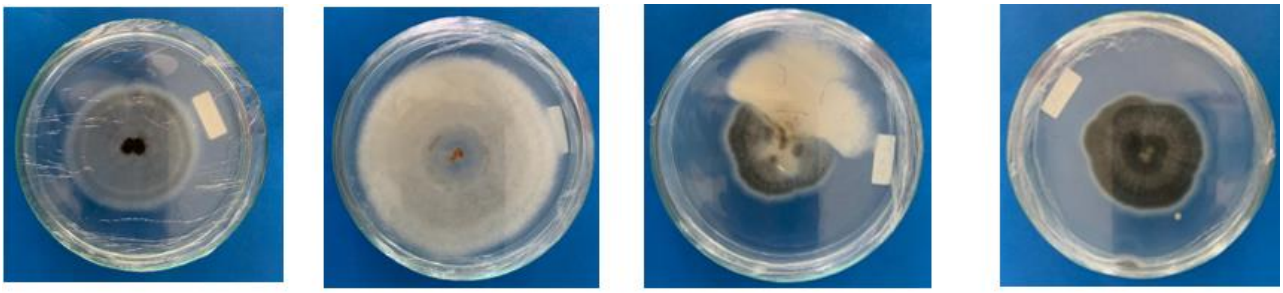

Figure 1 Neem (*Azadirachta indica* A.juss) Leaf Endophytic Fungi Isolate after incubation for 5 days, Description: (A) FDM 1, (B) FDM 2, (C) FDM 3, (D) FDM 4

3.2 Macroscopic and Microscopic Identification

The isolates obtained were identified macroscopically and microscopically. The results of macroscopic observations showed that FDM 2 had a relatively quicker growth rate than the other 3 isolates. Moreover, FDM 1, FDM 3, and FDM 4 isolates grew slightly slower.

Table 1. Macroscopic Character

Isolation Code	Macroscopic Characteristics
FDM 1	The colony color is slightly grayish white, the mycelium is spread, and the growth of fine colonies is wide
FDM 2	The colony color is white, the mycelium is spread, and the growth of the fine colonies is wide
FDM 3	The colony color is dark brown, the mycelium is spread, and the growth of fine colonies is wide
FDM 4	The colony color is blackish brown, the mycelium is spread, and the growth of fine colonies widens with fragments at the core of isolate growth that seems stiff and hardened.

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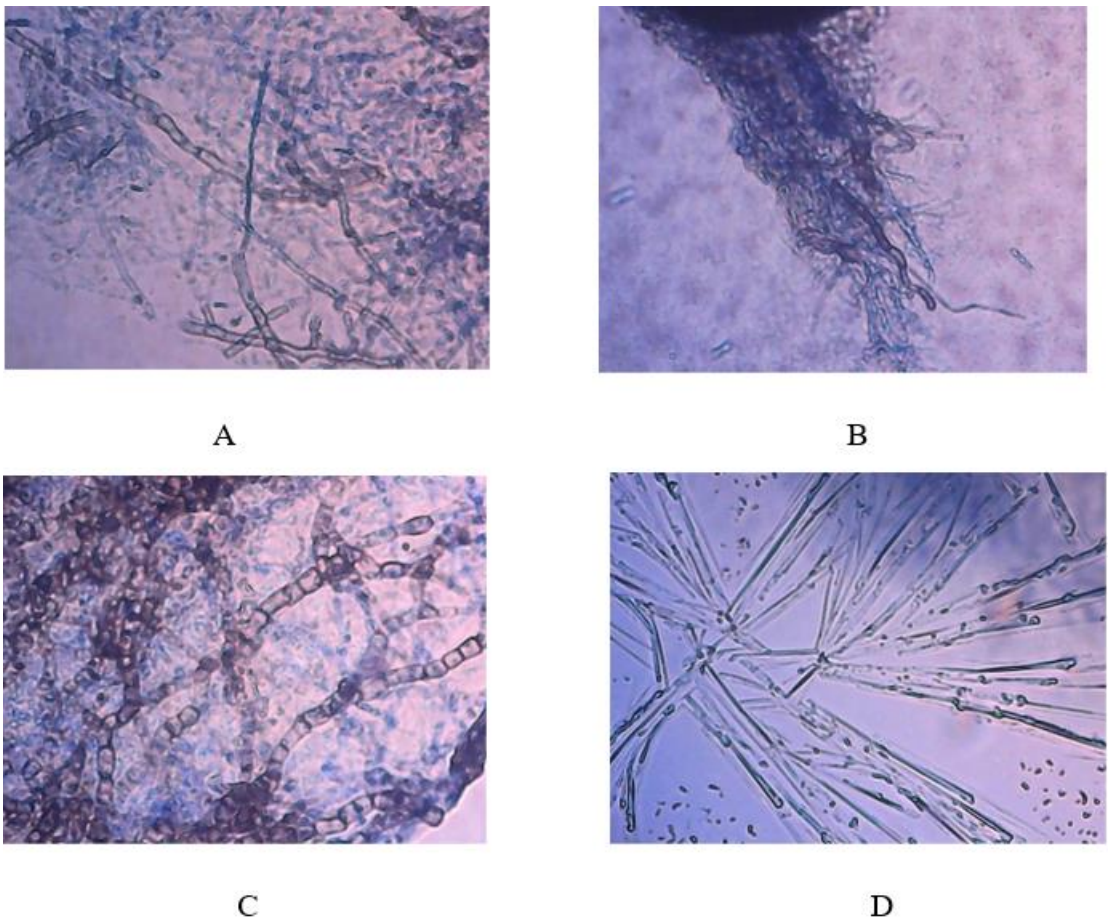

Figure 2. The Microscopic Results of Neem (*Azadirachta indica* A.juss) Leaf Endophytic Fungi Isolate, Description : (A) FDM 1, (B) FDM 2, (C) FDM 3, (D) FDM 4.

3.3 Results of Fermentation and Extraction of Neem Leaf Endophytic Fungi Isolates

The fungi were fermented using PDY media and the fermentation process was carried out with a rotary shaker at 150 rpm until the 10^{th} day because the fungi were in the stationary phase on the day. Meanwhile, secondary metabolites were formed during the stationary phase. The process was then stopped after the 10th day, and the extraction process was carried out. Also examined and used a fermentation time of 10 days for endophytic fungi [12].

The extraction process used a liquid-liquid extraction method with ethyl acetate as a solvent in a 1:1 (v/v) ratio. Meanwhile, ethyl acetate was used as a solvent because it is semi-polar, hence, it attracts both polar and non-polar compounds. The extraction results obtained 30 mg of FDM 1 extract, 40 mg of FDM 2, 50 mg of FDM 3 and 20 mg of FDM 4 each in 400 ml of EtOAc solvent.

3.4 Detection of Antioxidant Compounds

The extract's antioxidant compounds were detected using the TLC method. The eluent used was chloroform and methanol in a ratio of 4:1, the stains that appeared were then observed on 366 nm and 256 nm UV lamps. Subsequently, the sample was sprayed with a DPPH solution, and the yellow stain was measured for the Rf value.

The results of the preliminary test showed that the extract with activity against DPPH was FDM 2, indicated by the presence of yellow spots on the stain with RF values of 0.67 cm and 0.75 cm, respectively. Therefore,

the antioxidant measurement of the DPPH method using UV-Vis spectrophotometry was followed by extract with isolate code FDM 2.

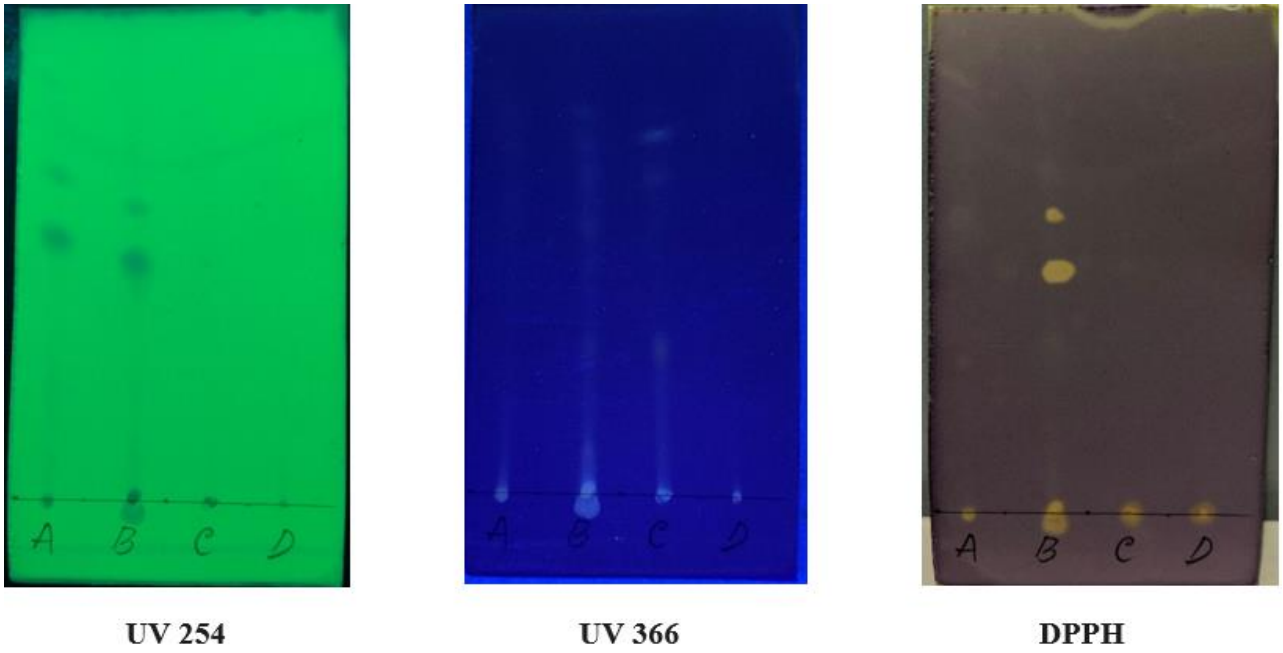

Figure 22 Chromatogram of the Fermented Neem (*Azadirachta indica* A.juss) Leaf Endophytic Fungi Extract, Description: (A) FDM 1, (B) FDM 2, (C) FDM 3, (D) FDM 4

3.5 Antioxidant Activity Test of FDM 2 Ethyl Acetate Extract with DPPH Method

FDM 2 isolates containing antioxidant compounds were fermented again using PDY media and extracted with ethyl acetate. The extract obtained was then tested for its antioxidant activity in vitro with the DPPH method using a UV-Vis spectrophotometer. Meanwhile, DPPH is a free radical that is stable at room temperature and often used to evaluate the antioxidant activity of several compounds or extracts of natural ingredients, specifically compounds with polar properties. Meanwhile, the principle of the DPPH method is that hydrogen atoms from an antioxidant compound turn the DPPH solution colorless, the solution is then measured using a spectrophotometer due to the formation of reduced DPPH [13].

The FDM 2 ethyl acetate extract was measured using a UV-Vis spectrophotometer at a wavelength of 516.8 nm. Furthermore, the IC50 for FDM 2 extract was 54.93 ppm and it was classified as having strong antioxidant activity. Specifically, a compound is a strong antioxidant if the IC_{50} value ranges from 51 - 100 ppm [14]. The measurement results of the antioxidant activity on FDM 2 extract are shown in Table 2.

Table. 2. Measurement results of Antioxidant Activity on FDM 2 Ethyl Acetate Extrac

No	Concentration (µg/mL)	Antioxidant Activity (%)	IC-50 Value (µg/mL)
1	15	11.35	54.93
2	30	32.87	
3	45	45.46	
4	60	53.31	
5	75	64.82	

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3.6 Molecular Identification

The primers used in this study were ITS 1 and ITS 4, and the amplification was carried out using the standard hotstar method with parameters: denaturation at 94 ^{o}C for 30 seconds, annealing at 50 ^{o}C for 30 seconds, extension at 72 ^{o}C, and then repeated in 38 PCR cycles. This method is in line with the principle of the hotstar method according to.

Table. 3 Amplification Primer

ITS 1	Primer Forward	5' – TCCGTAGGTGAACCTGCGG – 3'
ITS 4	Primer Reverse	5' – TCCTCCGCTTATTGATATGC – 3'

Figure 4 shows the DNA PCR products in form of ITS fragments after analysis with electrophoresis on 1% agarose gel. It shows that the DNA PCR ranged from 600 bp, and there was no non-specific priming, which was indicated by only 1 DNA band from the FDM 2 isolate. Meanwhile, stated that the ITS fragments for molds ranged from 565-613 bp.

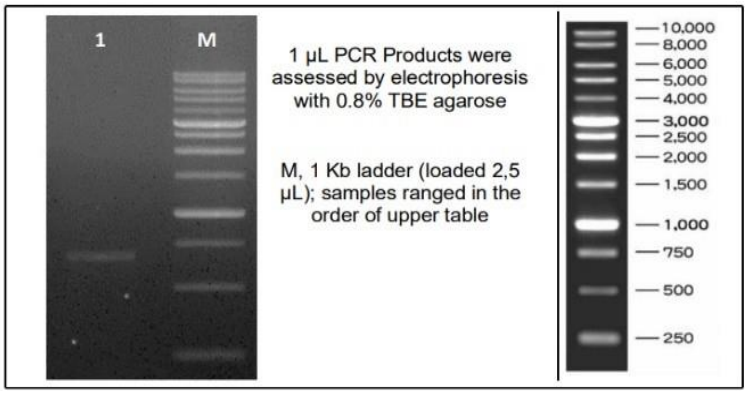

Figure 4. Gel Photo – PCR Products

The phylogenetic Tree-Neighbor Joining (NJ) of FDM 2 were formed using the Clustal X program and the Kimura method with two parameters. The phylogenetic scheme in Figure 5 shows similarity with *Lentinus squarosulus* isolates DQS74F with a percentage identity value of 99.55%. Meanwhile, molecular identification supports the macroscopic and microscopic characterization of FDM 2 fungal isolates. *Lentinus squarrosulus* is a wild mushroom with some interesting characteristics, such as rapid mycelium growth, as well as the potential to be used as food, and several other benefits. One of the most important potentials of this fungus is that it is a source of bioactive compounds [15]. Furthermore, previous study showed that *Lentinus squarrosulus* contains strong antioxidant compounds after in vitro testing [16].

Table 4. Top 10 Hit BLAST Results Against NCBI Database, Excluding Uncultured Sample Sequences

Deskripsi	Skor Maksimal	Skor Total	*Query Cover*	E-Value	Nilai *Identity*	Aksesi
Lentinus squarossulus isolate DQS74F	1229	1229	98%	0.0	99.55%	MZ735441.1
Lentinus squorossulus strain CH8-1	1216	1216	98%	0.0	99.11%	MG205584.1
Lentinus sp. GZMS-25	1214	1214	98%	0.0	99.11%	KX377592.1
Lentinus squorossulus strain 7-4-2	1214	1214	98%	0.0	99.11%	GU001951.1
Lentinus squorossulus strain 130343	1208	1208	98%	0.0	98,96%	MG208851.1
Lentinus squorossulus strain WCR1201	1205	1205	98%	0.0	98.68%	KT956127.1
Lentinus sp BAB-5060	1205	1205	98%	0.0	98.68%	KR155105.1
Lentinus sp S5	1205	1205	98%	0.0	98.68%	JN253598.1
Lentinus squorossulus isolate MEBP 0141	1201	1201	96%	0.0	99.54%	MT672530.1

Lentinus sp S3007	1199	1199	98%	0.0	98.81%	JQ868746.1

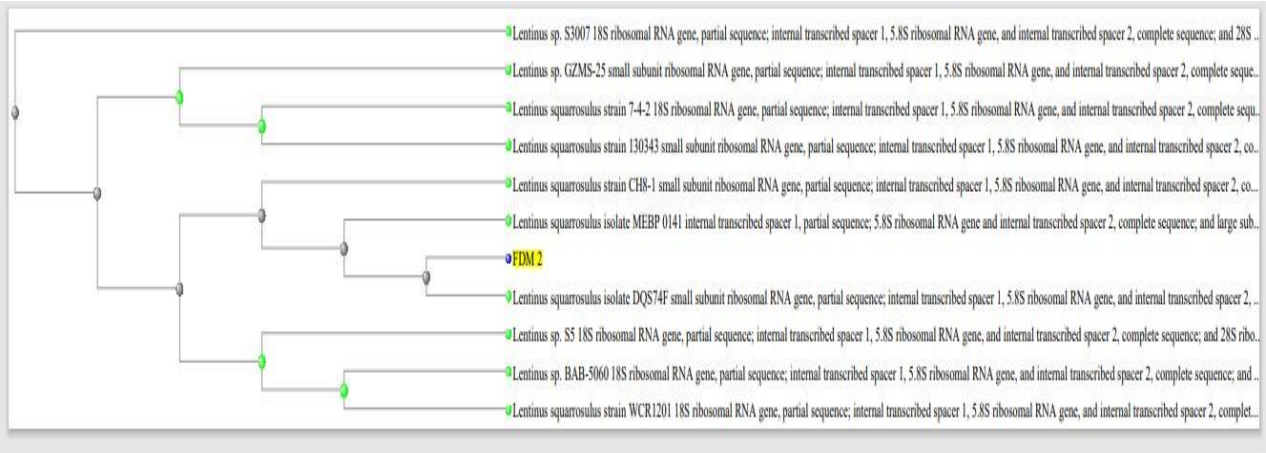

Figure 5 Phylogenetic Tree-Neighbor Joining (NJ) endophytic fungi FDM 2

The results of molecular identification showed that FDM 2 was similar to *Lentinus squarosulus* and these results support further testing of the antioxidant activity of endophytic fungus present in neem (*Azadirachta indica* A.juss) leaves which also have a strong antioxidant activity. Meanwhile, several studies also found that *Lentinus squarosulus* mushroom has a strong antioxidant activity, [17] and this is consistent with several references that Basidiomycetes mushrooms have medicinal benefits. *Lentinus squarrosulus* belongs to the division of Basidiomycetes fungi that have anti-tumor activity [18].

Furthermore, several studies reported that *Lentinus squarrosulus* mycelium extract contains substances that act as antioxidants, which proves that liquid fermentation makes it a source of antioxidant ingredients 16. *Lentinus squarrosulus* is an edible mushroom generally found in the wild and has not been cultivated on a large scale. It is also a white-rot saprophytic fungus, which lives on dead or decaying wood in forests. Meanwhile, mushroom's mycelia have been reported to have benefits as a good source of antioxidants, [19] Previous studies reported that *Lentinus squarrosulus* mycelium extract has antioxidant properties in vitro with no toxic effect, even at high doses [20].

However, *Lentinus. sp* fungi have been reported to be isolated from plant tissues, and stated that the fungus *Lentinus Sp.* can also be isolated from the leaves. The study isolated four endophytic fungi from the plant leaf and root tissue. *Daldinia sp.* and *Lentinus sp*. were later found in leaf tissue while *Rigidoporus sp.* and *Polyporales sp*. from root tissue [21].

4. CONCLUSION

Based on the results, endophytic fungi isolated from neem (Azadirachta indica A.juss) leaf, which was coded as FDM 2 have anti-oxidant activity with an IC50 of 54.93 ppm. Furthermore, the results of molecular identification based on the 18s rRNA gene of FDM 2 showed its similarities to Lentinus squarosulus with a similarity value of 99.55%.

5. CONFLICT OF INTEREST

The authors declare that they have no conflict of interests

6. REFERENCES

[1] Strobel G, Daisy B. Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiol

Mol Biol Rev. 2003;67(4):491-502. doi:10.1128/mmbr.67.4.491-502.2003

[2] Hung PQ, Annapurna K. Isolation and Characterization of Endophytic Bacteria in Soybean (Glycine Sp.). 2004;101:92-101.

[3] Taechowisan T, Lu C, Shen Y, Lumyong S. Secondary metabolites from endophytic Streptomyces aureofaciens CMUAc130 and their antifungal activity. Microbiology. 2005;151(5):1691-1695. doi:10.1099/mic.0.27758-0

[4] Aly AH, Debbab A, Proksch P. Fungal endophytes: Unique plant inhabitants with great promises. Appl Microbiol Biotechnol. 2011;90(6):1829-1845. doi:10.1007/s00253-011-3270-y

[5] Molina G, Pimentel MR, Bertucci TCP, Pastore GM. Application of fungal endophytes in biotechnological processes. Chem Eng Trans. 2012;27:289-294. doi:10.3303/CET1227049

[6] Visalakchi S, Muthumary J. Taxol (anticancer drug) producing endophytic fungi: An overview. Int J Pharma Bio Sci. 2010;1(3):1-9.

[7] Ramadass N, Subramanian N. Study of phytochemical screening of neem (Azadirachta indica). Int J Zool Stud 209 Int J Zool Stud. 2018;3(1):209-212.

[8] Balaji G, Cheralathan M. Experimental investigation of antioxidant effect on oxidation stability and emissions in a methyl ester of neem oil fueled DI diesel engine. Renew Energy. 2015;74(1):910-916. doi:10.1016/j.renene.2014.09.019

[9] Tang Z, Wang Y, Yang J, et al. Isolation and identification of flavonoid-producing endophytic fungi from medicinal plant Conyza blinii H.Lév that exhibit higher antioxidant and antibacterial activities. PeerJ. 2020;2020(4). doi:10.7717/peerj.8978

[10] Sithisarn P, Supabphol R, Gritsanapan W. Antioxidant activity of Siamese neem tree (VP1209). J Ethnopharmacol. 2005;99(1):109-112. doi:10.1016/j.jep.2005.02.008

[11] Smith A, Hursepuny A. Isolasi dan identifikasi jenis jamur pada ubi kayu (manihot esculenta crants.) dalam proses pembuatan ubi kayu hitam secara tradisional oleh masyarakat banda. BIOPENDIX J Biol Pendidik dan Terap. 2015;1(2):161.

[12] Kumala S, DwiYuliani K, Simanjuntak P, et al. Antimicrobial activity of secondary metabolites produced by endophytic fungi isolated from stems of Jati tree (Tectonagrandis L.F). Front Microbiol. 2016;6(Nov):2349-2353. doi:10.13040/IJPSR.0975-8232.6(6).2349-53

[13] Yang J, Chen J, Hao Y, Liu Y. Identification of the DPPH radical scavenging reaction adducts of ferulic acid and sinapic acid and their structure-antioxidant activity relationship. Lwt. 2021;146(December 2020):111411. doi:10.1016/j.lwt.2021.111411

[14] Jun , Yu , J ., fong , X ., Wan , C . S ., dan Yang C. T. Jun , Yu , J ., fong , X ., Wan , C . S ., dan Yang , C . T . 2011 . Comparison of Antioxidant Activities of Tabel 2 . 3 Ketentuan Kekuatan Antioksidan . 101-150 ppm 151-200 ppm Kekuatan Sangat kuat Kuat Sedang Lemah Sangat lemah. 2013;68(6):2122.

[15] Lau BF, Abdullah N. Bioprospecting of Lentinus squarrosulus Mont., an underutilized wild edible mushroom, as a potential source of functional ingredients: A review. Trends Food Sci Technol. 2017;61:116-131. doi:10.1016/j.tifs.2016.11.017

[16] Omar NAM, Abdullah S, Abdullah N, Kuppusamy UR, Abdulla MA, Sabaratnam V. Lentinus squarrosulus (Mont.) mycelium enhanced antioxidant status in rat model. Drug Des Devel Ther. 2015;9:5957-5964. doi:10.2147/DDDT.S90746

[17] Abdullah N, Mhd Omar NA, Kuppusamy UR, Abdulla MA, Sabaratnam V. Nutritional composition, antioxidant activities, and antiulcer potential of lentinus squarrosulus (Mont.) mycelia extract. Evidence-based Complement Altern Med. 2011;2011. doi:10.1155/2011/539356

[18] López-Legarda X, Arboleda-Echavarría C, Parra-Saldívar R, et al. Biotechnological production, characterization and in vitro antitumor activity of polysaccharides from a native strain of Lentinus crinitus. Int J Biol Macromol. 2020;164:3133-3144. doi:10.1016/j.ijbiomac.2020.08.191

[19] Mau JL, Chang CN, Huang SJ, Chen CC. Antioxidant properties of methanolic extracts from Grifola frondosa, Morchella esculenta and Termitomyces albuminosus mycelia. Food Chem. 2004;87(1):111- 118. doi:10.1016/j.foodchem.2003.10.026

[20] Ayimbila F, Siriwong S, Keawsompong S. Structural characteristics and bioactive properties of water- soluble polysaccharide from Lentinus squarrosulus. Bioact Carbohydrates Diet Fibre. 2021;26(May):100266. doi:10.1016/j.bcdf.2021.100266

[21] Maadon SN, Wakid SA, Zainudin II, et al. Isolation and identification of endophytic fungi from Uitm Reserve forest, Negeri sembilan. Sains Malaysiana. 2018;47(12):3025-3030. doi:10.17576/jsm-2018- 4712-12