



# A study of endophytic fungi *Neofusicoccum ribis* from Gandaria (*Bouea macrophylla* Griffith) as enzyme inhibitor, antibacterial, and antioxidant

Trisanti Anindyawati<sup>1\*</sup>, Praptiwi<sup>2</sup>

<sup>1</sup>Bioprocess Division, Research Center for Biotechnology, <sup>2</sup>Botany Division, Research Center for Biology, Indonesian Institute of Sciences, Jl. Raya Bogor Km.46, Cibinong 16911, Indonesia

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## ABSTRACT

This study aims to evaluate the potential of endophytic fungi isolated from Gandaria (*Bouea macrophylla* Griffith) as sources of enzyme inhibitor, antibacterial, and antioxidant and identify the chemical compounds of active extract. The endophytic fungi were identified as fungi closely related to the genus *Neofusicoccum ribis* based on sequence analysis of the 28S rDNA and internal transcribed spacer region. *N. ribis* was cultured on potato dextrose broth and extracted with hexane, ethyl acetate, and partition between methanol and ethyl acetate, respectively. Bioactivity screenings as enzyme inhibitor (acetylcholinesterase/acetylcholinesterase inhibitor (AChE) and  $\beta$ -glucosidase), antibacterial, and antioxidant were conducted by thin-layer chromatography-bioautography method. The chemical compounds were analyzed by gas chromatography-mass spectrometry (GC-MS). The results showed that the ethyl acetate extract of *N. ribis* had the activity as enzyme inhibitor for AChE and  $\beta$ -glucosidase. The minimum inhibitory concentration values of *N. ribis* extract against *Staphylococcus aureus* and *Escherichia coli* were in the range of 512–512  $\mu$ g/mL. Hexane extract had better antioxidant activity ( $IC_{50} = 479.66 \mu$ g/mL) than ethyl acetate extract ( $IC_{50} = 928.09–1102.60 \mu$ g/mL). The GC-MS analysis showed that 12 identified chemical compounds were found in the ethyl acetate extract of *N. ribis*.

## 1. INTRODUCTION

Plant has been known as a host of several endophytic fungi. The endophytic fungi defined as microorganisms that grow inside the plant without causing any harm to the host plant and they are living in a mutualistic symbiosis with their host [1,2]. Although the relationship between endophytes and their host differs each organism, endophytic fungi are an important component of microbial biodiversity [3]. Many endophytic fungi have the same or similar bioactive component because they live together with host plants for long period of time [4]. Some of endophytes may be producing bioactive substances that may have a relationship with host plant such as specific endophytes that produce taxol as an anticancer drug isolated from *Taxus brevifolia* [5]. They are also known to be producers of bioactive compounds. A number of interesting biological activities of endophytic

fungi, i.e., antioxidant, anticancer, immunomodulatory, antiviral, antitubercular, antiparasitic, and insecticidal activities [6], and a number of substances produced by endophytic fungi had been used in medicine, agriculture, and industry [7,8].

*Bouea macrophylla* Griffith or locally known as Gandaria in Indonesia is a tropical fruit plant from Southeast Asia. In Indonesia, it is distributed in North Sumatra, Java, Kalimantan, and Ambon. This plant belongs to the family of Anacardiaceae [9]. Gandaria fruit has significant amounts of nutrients and can be explored for the commercial purpose [10]. Methanolic extract of unripe fruits has high phenolics content, tannins and ascorbic acid, flavonoids, and flavonols in the ethanolic extract. The unripe fruits of *B. macrophylla* showed the highest antioxidant activity [11].

In this study, we reported the bioactivity of *N. ribis* extract isolated from Gandaria as sources of enzyme inhibitor, antimicrobial, and antioxidant and to identify the bioactive compounds of active extract by gas chromatography-mass spectrometry (GC-MS).

## 2. METHODS

### 2.1. Isolation and Identification of Endophytic Microbes

Fresh and healthy Gandaria plant was collected from Bogor Botanic Gardens, Indonesian Institute of Sciences in 2009. The healthy

\*Corresponding Author  
Trisanti Anindyawati,  
Research Center for Biotechnology,  
Indonesian Institute of Sciences,  
Jl. Raya Bogor Km. 46,  
Cibinong 16911, Indonesia.  
Phone: +62-21-875-4587,  
Fax: +62-21-875-4588.  
Email: [atrisanti@yahoo.com](mailto:atrisanti@yahoo.com)

twigs showing no disease symptoms were cut 1 cm length and washed under running tap water for 10 min. The surface of twigs was sterilized by soaking in 75% v/v ethanol for 1 min, 5.3% v/v sodium hypochlorite solution for 5 min, and then 75% v/v ethanol for 30 s, after that it was washed with sterilized water [12]. The sterilized twigs were dried on sterilized tissue towel before cutting longitudinally into two equal parts. Inside part of twigs was placed on sterilized medium of cornmeal malt extract agar supplemented with 50 mg/L chloramphenicol to suppress bacteria growth. The inside part of the twigs contacted with the surface of the agar media. All of the samples were incubated at 27°C for 2–3 weeks, and hyphae growing out from the twigs were cultured on potato dextrose agar (PDA). This was done several times to obtain pure isolate of endophytic fungi. The observation of fungi that growing up was carried out based on the color and surface. One of the pure endophytic fungi that excellent growth has been identified by Indonesian Culture Collection (InaCC) based on 28S rDNA (D1/D2 region) and internal transcribed spacer (ITS) rDNA. Primers used for polymerase chain reaction (PCR) amplification were NL-1 5'-GCA TAT CAA TAA GCG GAG GAAAAG-3' and NL-4 5'-GGT CCG TGT TTC AAG ACG-3' for 28S rDNA (D1/D2 region), meanwhile ITS 4 5'-TCC GCT TAT TGA TAT GC-3' and ITS 5 5'-GGA AGT AAA AGT CGT AAC AAG G-3' for ITS rDNA. PCR products were purified by polyethylene glycol precipitation method [13]. Sequence reading was done using a built-in software in the DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). Raw sequence trimming and editing were done using Mega 4 and BioEdit. Homology study was done by BLAST, using the Fasta-formatted query sequence. Phylogenetic tree construction and analyses were accomplished using Clustal X and NJ Plot.

## 2.2. Fermentation and Extraction

The pure isolate of endophytic fungus with excellent growth was cultured on PDA medium for 1 week. After that, approximately three small pieces of endophytic fungus ( $1 \times 1 \text{ cm}^2$ ) were transferred to 200 mL of potato dextrose broth in three of a 1000 mL Erlenmeyer flask. The fermentation process was done using shaking incubator for 12 days with a speed of 120 rpm. The duration of fermentation is reflected the stationary phase of the *N. ribis*. After fermentation completed, the biomass and media were centrifuged at 8000 rpm for 20 min to get the supernatant. The extraction process was done according to the modified method of Kumala *et al.* [14]. The supernatant from one Erlenmeyer was extracted with hexane (1:1), collected, and concentrated with a rotary evaporator (a). The supernatant from the second Erlenmeyer was extracted with ethyl acetate (1:1) (b), and the third Erlenmeyer was extracted with methanol (1:1) followed by partitioned with ethyl acetate (1:1) (c). Agitation process was done for 2 h with a speed of 120 rpm, then separated using a separating funnel, and allowed to stand some time until there was a complete separation. This process is performed 3 times. The extract was stored at 4°C until further analysis.

## 2.3. Acetylcholinesterase Inhibitor (AChE)

Five hundred units of AChE (Sigma) was dissolved in 500 mL Tris-hydrochloric acid buffer (0.05 mol/L, pH 7.8) and then added with 500 mg bovine serum albumin to stabilize the enzyme. Naphthyl acetate (150 mg) was dissolved in 40 mL ethanol and then diluted with 60 mL of distilled water. 50 mg of Fast Blue B salt was dissolved in 100 mL distilled water. 10  $\mu\text{L}$  of sample were transferred onto silica gel thin-layer chromatography (TLC) plate. After the

plate was absolutely dried, the plate was sprayed with 1-naphthyl acetate and blown with cold wind. When the plate dried, the plate was sprayed with enzyme solution, dried, and put on closed vessel or Petri dish with wet cotton to keep the humidity and incubate for 20 min at 37°C. After incubation period, the plate was sprayed with Fast Blue B solution. The extract showed AChE inhibitory activity would appear white, while inactive extract appears purple. The active compounds in extract were separated with mobile phase dichloromethane: methanol (10:1), and then, the same procedure was applied to the plate. The white bands indicated the active compounds as AChE inhibitor [15].

## 2.4. $\beta$ -Glucosidase Inhibitor

A 1000 units of  $\beta$ -D-glucosidase (sigma) was dissolved in 100 mL of buffer solution. Buffer solution was made by 10.25 g of sodium acetate in 250 mL distilled water; the pH was adjusted to 7.5 by adding acetic acid 0.1 M. The stock solution was kept at 4°C. 10  $\mu\text{L}$  of sample was transferred on to TLC plate and blown with cold wind then sprayed with enzyme solution. Plate was placed in closed Petri dish where the humidity was kept by putting wet cotton. The plate was incubated for 20 min at 37°C. After incubation period was completed, the plate was sprayed with a mixture of naphthyl-glucopyranoside solution and Fast Blue B salt solution (1:4) to give a purple background. The active extract indicated by change color to yellow. The active compounds in the extract were separated with mobile phase dichloromethane: methanol (10:1). The  $\beta$ -D-glucosidase inhibitory activity was done as the same procedure. The white bands indicated the active compounds in the extract [16].

## 2.5. Antibacterial Assay

The antibacterial test was done against *Staphylococcus aureus* InaCC B4 and *Escherichia coli* InaCC B5. Bacteria were culture overnight at 37°C in Mueller Hinton broth (MHB). Antibacterial screening was done by autobiography method at bacterial densities of  $10^8$  CFU/mL. 10  $\mu\text{L}$  of extract (100  $\mu\text{g}$ ) was transferred onto TLC plate (Silica gel GF<sub>254</sub>, Merck) and air-dried. The TLC plate then dipped into bacterial suspension, placed in sterile Petri dish, and incubated at 37°C for 18 h. The humidity in Petri dish was kept by putting sterile wet cotton. After incubation completed, the TLC plate was sprayed with iodinitrotetrazolium p-violet (INT). The active extract which suppresses bacterial growth was shown by clear zone formation. The active compounds in extract were separated with mobile phase dichloromethane: methanol (10:1). The antibacterial assay for the developed TLC-bioautography was carried out with the same procedure. The minimum inhibitory concentration (MIC) was determined by serial dilution method in 96-well microplate in triplicate. 100  $\mu\text{L}$  of 2-fold MHB medium was dispensed into each well on the 1<sup>st</sup> row. In each well, 100  $\mu\text{L}$  of extract (5120  $\mu\text{g}/\text{mL}$ ) in 10% dimethyl sulfoxide (DMSO) was dispensed and homogenized. Take out 100  $\mu\text{L}$  and put in the second row that has been fill with 100  $\mu\text{L}$  MHB media. This is done up to the last row. After that, 100  $\mu\text{L}$  bacterial suspension ( $10^6$  CFU/mL) was added in each well. Streptomycin and chloramphenicol were used as positive controls, while broth medium and DMSO were used as negative controls. The microplate was incubated at 37°C for 18 h. After incubation period, 10  $\mu\text{L}$  of INT was added into each well. Growth inhibition was determined by clear solution. MIC value was determined by the lowest concentration that shows no color change [17].

## 2.6. Antioxidant Assay

Antioxidant activity was determined by TLC-bioautography method. 10  $\mu\text{L}$  of extract (10  $\mu\text{g}/\text{mL}$ ) was transferred onto TLC plate. After air-dried, the plate was sprayed with 0.2% DPPH solution in methanol. The active extract was indicated by yellow spot with purple background after 30 min. To know the active compounds, the TLC plate was developed with dichloromethane:methanol (10:1) as mobile phase and air -dried following 30 min of DPPH sprayed, the yellow bands effected which indicated the active compounds. The active extract was further analyzed for its  $\text{IC}_{50}$  in triplicate. 100  $\mu\text{L}$  of sample in a concentration of 1000 ppm and catechin (positive control) was put in the well of 1<sup>st</sup> row. On the 2<sup>nd</sup>–8<sup>th</sup> rows, 50  $\mu\text{L}$  methanol was put in the well. The samples were diluted serially by taking out 50  $\mu\text{L}$  of sample from the 1<sup>st</sup> row and put in the 2<sup>nd</sup>–8<sup>th</sup> rows. After finish diluting the samples, 80  $\mu\text{L}$  of DPPH was added to each well and then incubated in the dark for 30 min, and the absorbance was determined at 517 nm with microplate reader (varioSCAN). The scavenging capacity ( $\text{SC}\%$ ) =  $[(A_0 - A_s)/A_0] \times 100\%$ , where  $A_0$  is the absorbance of reagent blank, while  $A_s$  is the absorbance of test sample.  $\text{IC}_{50}$  is the required concentration of the sample to scavenge 50% DPPH in certain time period [18].

## 2.7. GC-MS Analysis

Chemical compounds of ethyl acetate extract were carried out using GC-MS instrument (Agilent Technology GC System 6890N), equipped with capillary column HP-5MS (0.25 mm  $\times$  60 m  $\times$  0.25  $\mu\text{m}$ ). The instrument was set to initial temperature 80°C. Injector and detector temperature was 290°C. Helium with the purity of 99.9% was used as carrier gas at a flow rate of 1 mL/min, and the volume of injected sample was 3  $\mu\text{L}$ . The initial oven temperature was programmed at 80°C and increased up to 290°C at the rate of increase 15°C/min. The identification of the compounds was done by comparing mass spectra of the compounds with that of available mass spectral records (NIST library).

## 3. RESULTS AND DISCUSSION

### 3.1. Identification and Bioactivity of Strain

A newly isolated endophytic fungi from a twig of Gandaria plant were identified by means of PCR and DNA sequence analysis. Partial 28S rRNA (D1/D2 region) was compared with that of other plant fungi and found to be in the same cluster with *N. ribis*, *Botryosphaeria ribis*, and *Guignardia cryptomeriae* with 99% similarities. Since D1/D2 region could not differentiate *N. ribis* with *G. cryptomeriae*, further PCR amplified rDNA ITS analysis which confirms these endophytic fungi to be 99.9% identical with *N. ribis*.

Bioactivity of *N. ribis* extract was done by TLC-bioautography method. This assay is the simplest guided bioassay to discover new bioactive compounds from natural product [19]. TLC-bioautography is facilitating the localization and target-directed isolation of active compounds of extract [20]. Extract of endophytic fungi isolated from *B. macrophylla* was tested for its bioactivities, i.e., AChE and  $\beta$ -glucosidase inhibitor, antibacterial against *S. aureus* and *E. coli*, and also antioxidant as DPPH radical scavenger.

### 3.2. TLC-Bioautography - AChE Inhibitor

The AChE inhibitory activity of endophytic fungi extracts was done by TLC bioautography. The test relies on the cleavage by AChE of 1-naphthyl acetate to form 1-naphthol, which in turn reacts with Fast Blue B salt to give a purple-colored diazonium dye [21,22].

Enzyme inhibitors block the formation of  $\alpha$ -naphthol and there is no purple coloration on TLC plates [22]. This method is quick in giving information about the activity and localization of active compounds in the extract [21] and rapid identification of a large number of AChE inhibitors [22]. The active chemical compounds in the extract as acetylcholinesterase inhibitor showed white spots. The extract of endophytic fungi *N. ribis* showed white spots that indicate that these compounds have AChE inhibitory activity [Figure 1].

### 3.3. TLC-Bioautography - $\beta$ -Glucosidase Inhibitor

Glucosidase inhibitors are responsible for disruption of the activity of glucosidase [23] and potentially useful as antidiabetic, anti-obesity, antiviral, antiadhesive, antibacterial, or antimetastatic agents [24]. The result for TLC-bioautography of endophytic fungi *N. ribis* for  $\beta$ -glucosidase inhibitor is shown in Figure 2 that the extract contains compounds with  $\beta$ -glucosidase inhibitor activity.

### 3.4. TLC-Bioautography - Antibacterial Activity

Screening of antibacterial activity of *N. ribis* extract against *S. aureus* and *E. coli* is shown in Figure 3. It was indicated that extract derived



**Figure 1:** Thin-layer chromatography bioautography for acetylcholinesterase inhibitor (AChE). White bands indicate active compounds as AChE inhibitor



**Figure 2:** Thin-layer chromatography bioautography of endophytic fungi *Neofusicoccum ribis* for  $\beta$ -glucosidase inhibitor. White bands indicate the compounds as  $\beta$ -glucosidase inhibitor

from methanol partitioned with ethyl acetate (C) was the most active extract against *S. aureus* compared to A and B, while extract B and C also active against *E. coli*.

The growth inhibition by the extract was indicated by clear zone formation after sprayed with INT. TLC plate as a background changes its color to purple after sprayed with INT. The purple color is caused by the conversion of INT to intensely colored formazan by the dehydrogenases enzyme of living microorganisms [25]. The interaction between viable microorganisms and INT caused a color change of INT from faint yellow to red–purple color [19]. The result of antibacterial activity showed that extracts B and C have several compounds that suppressed the growth of *S. aureus* and *E. coli*. The active compound that suppressed both bacteria has similar Rf value. This indicated that the compound might have a broad spectrum in suppressing bacterial growth. The results are shown in Table 1 that all of the extracts had MIC value >512 µg/mL against *E. coli* and only extract C had the MIC value 512 µg/mL. Extract with MIC value from 500 to 1000 µg/mL was considered as weak antibacterial activity [26].

### 3.5. TLC-Bioautography - Antioxidant Activity

Antioxidant activity assay used in this study was DPPH radical scavenging activity. DPPH radical scavenging assay is a quick, reliable, and reproducible for screening *in vitro* antioxidant of crude extract and pure compound. DPPH has the ability to donate hydrogen [19]. The antioxidant compound of extract reduced the capacity of DPPH radical by the decrease in absorbance [27]. The IC<sub>50</sub> value of extract A was the lowest compared to extracts B and C. It was indicated that extract A had better antioxidant activity than extracts B and C [Table 2].

### 3.6. Analysis of Chemical Compounds of *N. ribis* by GC-MS

There are 12 active compounds identified by GC-MS analysis from *N. ribis* ethyl acetate extract: Undecane, cyclopentasiloxane, dodecane, 1-tetradecene, phenol, 1-hexadecene, 1-octadecene, N,N-diethyl-N',N'-diphenyl-6-pyrrol-1-yl-[1,3,5]triazine-2,4-diamine, 1-docosene, N,N-diethyl-N',N'-diphenyl-6-pyrrol-1-yl-[1,3,5]triazine-2, 4- diamine, and 1-nonadecene [Table 3]. The peak values identified in above extract are shown in Figure 4. Most of the identified compounds have been reported to possess interesting biological activities. Dodecane reported as antifungal/antibacterial [28,29]. 1-Octadecene and 1-hexadecene have been reported as antibacterial/antifungal, antioxidant, and anticancer [30]. Docosene was also reported has the ability as antifungal [31], while phenol has been reported as antioxidant, anticarcinogenic, and anti-inflammatory [32].

## 4. CONCLUSION

This study revealed that endophytic fungi of *N. ribis ethyl acetate extract* isolated from Gandaria plant contain 12 chemical compounds, some of them have biological activities. The extract contains chemical compounds with antibacterial, antioxidant, and enzyme inhibitors, especially acetylcholine esterase inhibitor and β-glucosidase inhibitor. The *N. ribis* extract against *S. aureus* and *E. coli* was in the range MIC values 512–>512 µg/mL, while hexane extract has better antioxidant activity (IC<sub>50</sub> = 479.66 µg/mL) than ethyl acetate extract (IC<sub>50</sub> = 928.09–1102.60 µg/mL). The activity as enzyme inhibitors is present

**Table 1:** MIC of *N. ribis* extract

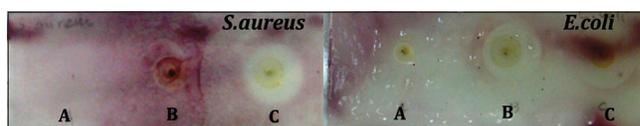
No	Extract	MIC (µg/ml)	
		<i>S. aureus</i>	<i>E. coli</i>
1	A	>512	>512
2	B	>512	>512
3	C	512	>512

MIC: Minimum inhibitory concentration, *N. ribis*: *Neofusicoccum ribis*

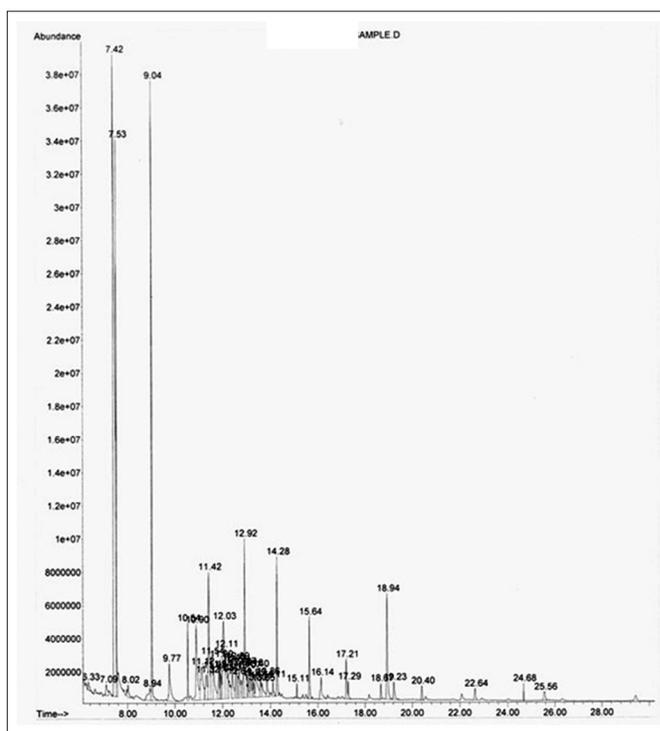
**Table 2:** IC<sub>50</sub> value of *N. ribis* extract

No	Extract	IC <sub>50</sub> (µg/ml)
1	A	479.66
2	B	928.09
3	C	1102.60

*N. ribis*: *Neofusicoccum ribis*



**Figure 3:** Thin-layer chromatography bioautography of *Neofusicoccum ribis* extract for antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*



**Figure 4:** Gas chromatography-mass spectrometry analysis of chemical compounds

only in ethyl acetate extracts. Further, isolation and purification of bioactive compounds need to be done.

**Table 3:** GC-MS Identification

No	Retention time (min)	Area (%)	Library/ ID	Quality
1	7.09	0.48	Undecane	96
2	7.42	29.10	Cyclopentasiloxane	95
3	8.02	0.43	Dodecane	95
4	9.77	1.84	1-Tetradecene	99
5	10.90	3.23	Phenol	95
6	11.42	3.61	1-Hexadecene	98
7	12.93	4.14	1-Octadecene	99
8	14.28	2.38	1-Octadecene	96
9	15.12	0.21	N, N-DIETHYL-N', N'-DIPHENYL-6-PYRRO L-1-YL-[1,3,5]TRIAZINE-2,4-DIAMINE	95
10	17.21	0.95	1-Docosene	99
11	18.67	0.32	N, N-DIETHYL-N', N'-DIPHENYL-6-PYRRO L-1-YL-[1,3,5]TRIAZINE-2,4-DIAMINE	95
12	19.23	0.66	1-Nonadecene	97

GC-MS: Gas chromatography-mass spectrometry

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