Isolation, identification and antimicrobial activity of secondary metabolites of endophytic fungi from annona leaves (*Annona squamosa L.*) growing in dry land

Antonius R B Ola¹,², Yoseph Sugi³, Caterina S Lay¹,²

¹Chemistry Department, Faculty of Science and Engineering, University of Nusa Cendana, Kupang 85118 Indonesia
² Integrated Center Research (Bioscience) Laboratory, University of Nusa Cendana, Kupang 85118 Indonesia
³Faculty of Science and Engineering, University of Nusa Cendana, Kupang 85118 Indonesia

E-mail: antonius.ola@staf.undana.ac.id

Abstract. Research on isolation, identification, and antimicrobial activity of secondary metabolites of endophytic fungi from leaves of *Annona squamosa* growing in dryland had been undertaken. The work includes the isolation of endophytic fungi, cultivation, and extraction of fungal extract and identification of chemical metabolites together with the antibacterial test. The pure colony of endophytic fungi was grown on solid rice media using 1 L Erlenmeyer flask. The grown fungi were extracted with ethyl acetate. The ethyl acetate crude extract was then further subjected to chemical analysis and its antibacterial properties. Endophytic fungi species was identified as *Aspergillus niger* based on macroscopic and microscopic analysis. Analysis LC-MS/MS had revealed the presence of five metabolites including ephedradine A, ergosine, Ia, mudanpioside H, and trichosanic acid. The extract showed strong inhibition against *Staphylococcus aureus* with diameter of zone inhibition of 16.1 mm and moderate inhibition against *Escherichia coli* 0175H7 and *Salmonella enteritidis* ATCC 6939 with the observed diameter of zone inhibition of 9.6 mm and 11.3 mm, respectively.

1. Introduction
One of the important sources of bioactive compounds is from microbes including endophytic fungi that grow inside plant tissue such as leaves, flowers, seeds, twigs, stems, and roots without causing tissue damage in the host plant [1]. These fungi are rich in secondary metabolites and have been reported to provide and produce several lead drugs and drugs to cure deadly diseases such as cancer and bacterial infections [2]. Examples of endophytic fungus bioactive compounds that have been isolated from plants were equisetin, epi equisetin and beauvericin produced by *Fusarium equiseti* isolated from *Piper niger* leaves [3]. Another example was neosartorin, a potent antibiotic compound against multi-resistant bacteria without any significant toxicity observed, together with (-) Palitantin.
from endophytic fungi *Aspergillus fumi-gatiafinis* isolated from the leaf of the *Tribulus terrestris* (Zygophyllaceae) [4,5].

Several works regarding the bioactive fungal metabolites isolated from plants growing in Timor Island have been reported. The endophytic fungi *Xylaria* sp isolated from *Curcuma xanthoriza* leaves produced arugosin J, xylarugosin, and resacetophenone [6]. While the endophytic fungi *Diaporthe melonis* isolated from annona twigs produced diaporthemin A and B together with flavomannin dimethyl ether [7]. *Aspergillus flavus* isolated from the medicinal plant *Catharanthus roseus* growing in Timor Island was currently reported to accumulate high content of kojic acid, which has wide application in cosmetics and pharmaceutical industries [8]. In continuation of our work on finding fungal bioactive metabolites from Timor Island, we evaluated antibacterial extract from the endophytic fungi *Aspergillus niger* associated with *Annona* leaves.

2. Material and Methods

2.1 Material

The endophytic strains selected for the obtainment of crude extracts were isolated from the fresh leaf of *Annona squamosa* L located at Timor Island East Nusa Tenggara, Indonesia. Samples of leaves were transported to the laboratory in sterile baggage.

2.2 Isolation of endophytic fungi

The healthy leaf samples were washed several times using sterile distilled water and followed by immersing the tissues in 70% alcohol for 30 s and sterile distilled water for eliminating epiphytic microorganisms. The leaves were cut into 0.5 cm² pieces and then transferred to Petri dishes containing potato dextrose agar supplemented with chloramphenicol (0.02 g) to suppress bacterial growth. After five days, all fungal colonies were isolated, purified, and maintained in PDA.

2.3 Cultivation and extraction of secondary metabolite

In order to get secondary metabolites for antimicrobial activity screening, the pure culture of every endophytic fungus was grown in 100 g of rice media and incubated for thirty days. After reaching its stationary phase, the fungi were extracted with ethyl acetate. Ethyl acetate was removed by rotary evaporator. The crude extract was analyzed for its chemical profile by using HPLC and LC-MS/MS.

2.4 Antibacterial assay

The paper disk method used to screen the antimicrobial activity of the EtOAc endophytic fungus extracts. The micro-organisms were one Gram-positive bacteria *Staphylococcus aureus* camp. and two Gram-negative bacteria *Escherichia coli* 0175H7 and *Salmonella enteritidis* ATCC 6939. The test microorganisms were cultivated in the test tubes comprising 2 g/100 ml of nutrient broth and incubated for 24 h at 37°C. Turbidity was adapted to that of a standard for barium sulfate (0.5 McFarland). Paper disks were also inoculated with aquades (10 µl) as the negative control, tetracyclin (30 µg) as the positive control. On the surface of the medium containing bacteria test strain, each 10 µl of extracts were pipetted into 0.66 cm sterile paper disk. All plates were incubated for roughly 24 hours at 37°C. Zones of inhibition were evaluated and recorded. Antibacterial activity screening was repeated twice.

3. Result and Discussion

A white and black strain of endophytic fungi was isolated from the leaf of *Annona squamosa*. Based on the macroscopic and microscopic characteristics and after comparison with endophytic fungus morphology according to [9], the isolated endophytic fungus was identified as *Aspergillus niger*.  


Figure 1. *Aspergillus Niger* from old annon leaves, (a) Front view and (b) Rearview

Figure 1 showed that the black and white endophytic fungi have been successfully isolated and purified from the leaves of the mature annon leaves.

Fungus cultivation was performed for 3 weeks so that the fungus was able to grow until its stationary phase was reached. The endophytic fungi were then extracted with ethyl acetate and left for two nights before filtration. The solvent was removed under vacuum using a rotary evaporator. The crude extract was then analyzed with HPLC and LC-MS for its chemical profile (Figures 2 and 3). In addition, the extract was also evaluated for its antibacterial properties.

The chemical content of fungal extract was performed with HPLC using C-18 analytical column at a flow rate of 0.5 mL/minute for 15 minutes and detection at 204 nm.

Figure 2. HPLC chromatogram

Figure 3. Chromatogram LC-MS / MS injection volume 1.00 µl
Five metabolites were able to be identified using LC-MS/MS method as shown in Table 1.

**Table 1.** Chemical components of *Aspergillus niger* (black endophytic fungi) ethyl acetate extract

| No | Retention time (Minute) | Weight (m/z) [M+H+] | Compound Weight (Dalton) | Compound |
|----|-------------------------|---------------------|--------------------------|----------|
| 1  | 6.04                    | 493,2814            | 492,27366                | Ephedradine A |
| 2  | 5.10                    | 548,2873            | 547,27947                | Ergosine   |
| 3  | 6.86                    | 379,2506            | 378,24062                | Ia        |
| 4  | 6.02                    | 639,1695            | 638,17921                | Mudanpioside H |
| 5  | 6.68                    | 279,2359            | 278,22458                | Trichosanic acid |

**a. Identification of Ephedradine A**

![Identification of Ephedradine A](image)

**Figure 4.** LC-MS / MS chromatogram of Ephedradine A

The structure of ephedradine A was shown in Figure 5.

![Structure of Ephedradine A](image)

**Figure 5.** Structure of Ephedradine A compounds.
b. **Identification of Ergosine**

Component name: Ergosine

![Ergosine LC-MS/MS chromatogram](image)

**Figure 6.** LC-MS / MS chromatogram of Ergosine

Based on the results of the LC-MS / MS chromatogram, the structure of Ergosine was shown in Figure 7.

![Ergosine structure](image)

**Figure 7.** Structure of Ergosine compounds.

c. **Identification of Ia**

Component name: Ia

![Ia LC-MS/MS chromatogram](image)

**Figure 8.** LC-MS / MS chromatogram of Ia

Based on the results of the LC-MS / MS chromatogram, the structure of Ia compounds was shown in Figure 9.
d. Identification of Mudanpioside H

Component name: Mudanpioside H

Figure 9. Structure of Ia compounds.

Based on the results of the LC-MS / MS chromatogram of Mudanpioside H, the structure of Mudanpioside H was shown in Figure 10.

Figure 10. LC-MS / MS chromatogram of Mudanpioside H

Figure 11. Structure of Mudanpioside H compounds.
Identification of Trichosanic acid

Component name: Trichosanic acid

Figure 12. LC-MS / MS chromatogram of Trichosanic acid bioactive compounds

Based on the results of the LC-MS / MS chromatogram, the structure of Trichosanic acid compounds is shown in Figure 13.

Figure 13. Structure of Trichosanic acid compounds.

The ethyl acetate fungal extract was further evaluated for its antibacterial properties against *S. aureus*, *E. coli* and *Salmonella enteritidis* with tetracycline as positive control and sterile distilled water as the negative control. The result was shown in Table 2.

| No | Type of Bacteria                  | Aspergillus Niger ethyl acetate extract (10 µg) | Tetracycline (positive control) (30 µg) | Aquades (negative control) (10 µL) |
|----|-----------------------------------|-----------------------------------------------|---------------------------------------|----------------------------------|
| 1  | *Staphylococcus aureus camp*      | 16,1                                          | 26,8                                  | 0,6                              |
| 2  | *Escherichia coli 0175H7*         | 9,6                                           | 28,7                                  | 0,6                              |
| 3  | *Salmonella enteritidis ATCC 6939*| 11,3                                          | 24,5                                  | 0,6                              |

Gram-negative and gram-positive bacteria used in this study had a concentration of $10^8$ cfu/mL. The fungal extract was found to inhibit bacterial growth at a concentration of 10 µg. Of all the identified components, Mudanpioside H was probably responsible for the antibacterial activity of the extract as it was previously reported to have moderate antibacterial activity [9]. However, further fractionation and isolation of pure metabolite from the fungal extract should be undertaken.
References

[1] Namasivayam S K R, Swetha R and Srivatsan K V 2014 Evaluation of potential biological activities of metabolites from endophytic fungi residing in leaves of *Azadirhacta indica* *Int. J. ChemTech. Res.* **5** 3116–21

[2] Yu H, Zhang L, Zheng C, Guo L, Li W and Sun L 2010 Recent developments and future prospects of antimicrobial metabolites produced by endophytes *Microbiol Res.* **165**(6) 437–49

[3] Ola A R B, Amal H A, Wen H L, Victor W and Abdessamad D 2014 Absolute configuration and revision of the structure of lateritin *Tetrahedron Letters.* **55** 3147–50

[4] Ola A R B, Amal H A, Ilka Z, Alexandra H, Attila M, Matthias K, Heike B O, Tibor K, Peter, Proksch and Abdessamad D 2014 Absolute configuration and antibiotic activity of neosartorin from the endophytic fungus *Aspergillus fumigatiaffinis* *Tetrahedron Letters.* **55** 1020–23

[5] Ola A R B, Tawo B D, Belli H L L, Proksch P, Tommy D and Hakim E H 2018 A new antibiotic polyketide from the endophytic fungi *Aspergillus fumigatiaffinis* *Natural Product Communications* **13**(12) 1934578X1801301202

[6] Hammerschmidt L, Ola A, Werner E G M, WenHan L, Attila M, Tibor K, Peter P and Amal H A 2015 Two new metabolites from the endophytic fungus *Xylaria* sp. isolated from the medicinal plants *Curcuma Xanthorrhiza* *Tetrahedron Letters.* **56** 1193–97

[7] Ola A R B, Heike B O, Abdessamad D, Peter P and Amal H A 2014 Dihydroanthracenone metabolites from the endophytic fungus *Diaporthe melonis* isolated from *Annona squamosa* *Tetrahedron Letters.* **55** 3133–36

[8] Ola A R B, Metboki G, Lay C S, Sugi Y, Rozari P D, Darmakusuma D and Hakim E H 2019 Single production of kojic acid by *Aspergillus flavus* and the revision of flufuran *Molecules* **24**(22) 4200

[9] An R.-B, Kim H.-C, Lee S.-H, Jeong G.-S, Sohn D.-H, Park H, Kwon D.-Y, Lee J. H and Kim Y.-C 2006 A new monoterpene glycoside and antibacterial monoterpene glycosides from *Paeonia suffruticosa* *Archives of Pharmacal Research* **29**(10) 815