

# Antimicrobial Compound from Endophytic *Pseudomonas azotoformans* UICC B-91 of *Neesia altissima* (Malvaceae)

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## ABSTRACT

Centrifugated extract of endophytic *Pseudomonas azotoformans* strain UICC B-91 has been observed for its antimicrobial activity against *Escherichia coli* ATCC 8739, *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 6583, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 25241, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis* ATCC 19659 and *Candida albicans* ATCC 10231, with minimum concentration in the range 250 µg/mL. But, the novel compound of the bioactive substances which relevant for antimicrobial activity have not been described. Further fractionation and identification of the extract by thin layer chromatography (TLC) and liquid chromatography mass spectrophotometry (LCMS-MS) detected several compound as futoamide (C<sub>18</sub>H<sub>23</sub>NO<sub>3</sub>), gentialutine (C<sub>9</sub>H<sub>11</sub>NO), gentiabetine (C<sub>9</sub>H<sub>11</sub>NO<sub>2</sub>), 1-[(2E,4E)-2,4-decadienyl]pyrrolidine (C<sub>14</sub>H<sub>23</sub>NO), lycopodine (C<sub>16</sub>H<sub>25</sub>NO) and dihydrolycopodine (C<sub>16</sub>H<sub>27</sub>NO). The majority compounds containing CHON elements with molecular weight approximately 100-450 Da.

**Keywords:** Alkaloid, Antimicrobial compound, Extraction, *P. azotoformans*.

## INTRODUCTION

*P. azotoformans* strain UICC B-91 was isolated from *Neesia altissima* Blume, traditional medicinal plant endemic from Indonesia<sup>1</sup> that effective against gonorrhoeae<sup>2</sup> due to *Neisseria gonorrhoeae* infection. But there is no publication research on bioactive natural compound from *N. altissima*, yet. Endophytic microorganism has genetic diversity due to adaption to host environment and might be transfer gen from host, that relate to the bioactive compound produced.<sup>3</sup> Previous research has been observed the antimicrobial activity from centrifugated extract of endophytic *P. azotoformans*, against several bacteria and *C. albicans*.<sup>4</sup> with notice result on its anticandidal activity. For further observation, the antimicrobial compounds were identified by mass spectrophotometry.

Several antimicrobial compounds which have been identified from *P. azotoformans* was rhamnolipid that able to inhibit various species of *Bacillus*,<sup>5</sup> 1-acetyl-beta-carboline that able to inhibit MRSA,<sup>6</sup> 2-aminofenol, 5-methyl-2,4-imidazolidinedione, 1,4-benzenediamine,N-(1-methylethyl)-N-phenyl, pirrol[1,2-a]pirazin-1,4-diona, pirrol[1,2-a]pirazin-1,4-diona,hexahydro-3-(phenylmethyl), and hexadecenoic acid that able to inhibit *Neisseria meningitidis* and *S. aureus*.<sup>7</sup> Thus research encourage us to explore antimicrobial compound especially alkaloids. Alkaloid exhibit broad bioactivities, potentially new type of broad spectrum natural antimicrobial compound, and low tendency to produce resistance.<sup>8,9</sup>

This study sought to explore antimicrobial activity on extract of *P. azotoformans* without centrifugation on extraction and observed the chemical compound from endophytic *P. azotoformans* as antimicrobial compound.

## MATERIALS AND METHODS

### Microorganisms

*P. azotoformans* strain UICC B-91 used in this research was deposit culture of Universitas Indonesia of Culture Collection. Microorganisms for antibacterial susceptibility screening were *E. coli* ATCC 8739, *B. cereus* ATCC 10876, *B. subtilis* ATCC 19659, *P. aeruginosa* ATCC 15442, *S. typhimurium* ATCC 25241, *S. aureus* ATCC 6583, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231.

### Extraction of antimicrobial compound

The extractions were proceeded by two culture mediums as Nutrient Broth (NB) and Rehydrated Broth (RB). The RB compositions are yeast extract, peptone, and MgSO<sub>4</sub>. Fermentations and various extractions treatments were described on Figure 1. The extracts then concentrated by rotary evaporator on 40°C. The resulted was crude extract (Table 1). Crude extract then separated through column chromatography using silica gel 60 G F254.

### Antimicrobial activity

Antimicrobial activity analysis were regarding to disc diffusion.<sup>10,11</sup> The antimicrobial activity was measured by diameter of clear zone minus diameter of disc diffusion.

### Thin Layer Chromatography (TLC)

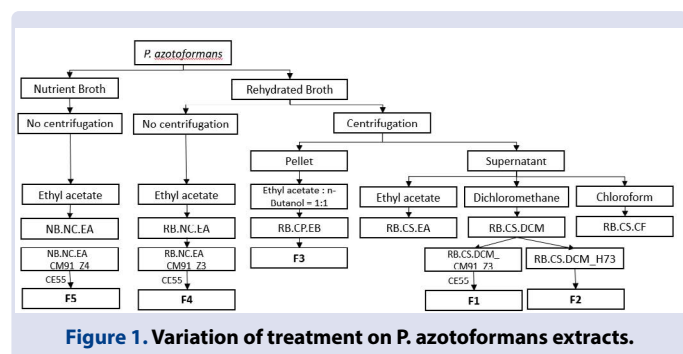
TLC analytical, TLC-bioautography and semi-preparative separation were performed on aluminium silica TLC F254 plates. The TLC plates were developed with mobile phase chloroform: methanol (9:1) (F1, F3, F4, and F5) and hexane: ethyl acetate (7:3) (F2). Screening for antimicrobial compound conducted by TLC-bioautography.<sup>12</sup> Positive result reflected on clear zone developed by Rf. Further TLC-separation on clear zone-Rf were developed with chloroform: ethyl acetate (5:5) (F1, F4, and F5).

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**Table 1. Description of the research.**

| Extract   | Antimicrobial activity    | Structure identification | Fraction |
|-----------|---------------------------|--------------------------|----------|
| RB.CS.EA  | Ref in press <sup>4</sup> | n.d.                     |          |
| RB.CS.CF  | Ref in press <sup>4</sup> | n.d.                     |          |
| RB.CS.DCM | Ref in press <sup>4</sup> | This publication         | F1, F2   |
| RB.CP.EA  | Ref in press <sup>4</sup> | This publication         | F3       |
| RB.NC.EA  | This publication          | This publication         | F4       |
| NB.NC.EA  | This publication          | This publication         | F5       |

n.d. = not determined

**Figure 1. Variation of treatment on *P. azotoformans* extracts.**

## Identification and structure determination

The analysis on fractions was performed by LCMS/MS using XEVO G2-XS QT of mass spectrophotometer. The ionisation was using Electro Spray Ionisation (ESI), with scan range 50 to 1,200 m/z. Ultra-performance liquid chromatography (UPLC) was performed using a Waters Acquity UPLC I-Class system. Chromatographic separation was carried out with ACQUITY UPLC® BEH C8 (2.1 x 100 mm, 1.7 µm) on 40°C. The mass-to-charge ratio (m/z) values and retention time analysed was identified using UNIFI software. For candidate mass, the values were analysed using databases in Metfrag and PubChem. The identified compounds were also compared using *Pseudomonas aeruginosa* Metabolome Database by University of Maryland School of Pharmacy (PAMDB), Natural Product Activity and Species Source Database (NPASS), and The Dictionary of Alkaloid.<sup>13</sup> The <sup>13</sup>C-NMR was performed by NMR Agilent with DD2 system console and 125 MHz reading. Chloroform was used as solvent (DDCl<sub>3</sub>) and tetramethylsilane (TMS) as chemical shifting reference.

## RESULT

### Antimicrobial activity on crude extract

The result of the antimicrobial activity of ethyl acetate extract of *P. azotoformans* endophytic are described on Table 2. The *in vitro* antimicrobial activity was shown on both crude ethyl acetate extract on gram negative bacteria as *E. coli* ATCC 8739, *S. typhimurium* ATCC 25241, *P. aeruginosa* ATCC 15442; gram positive bacteria as *B. subtilis* ATCC 19659, *B. cereus* ATCC 10876, *S. aureus* ATCC 25923, *S. aureus* ATCC 6583; and yeast of *C. albicans* ATCC 10231. The range of minimum inhibition concentration of NB.NC.EA was on 50,000 – 5,000 µg/mL and for RB.NC.EA, it goes lower until 1,000 µg/mL. Crude extract of RB.NC.EA also has widest clear zone diameter than NB.NC.EA, especially on *P. aeruginosa* and *C. albicans*. The minimum inhibition concentration of *C. albicans* on RB.NC.EA was 1,000 µg/mL. NB.NC.EA was better on inhibiting growth of gram-positive bacteria than gram-negative bacteria. Tetracycline (100 µg/mL) as broad range antibiotic was given clear zone as positive result of inhibition on gram positive and negative bacteria. Ketoconazole (10,000 µg/mL) as

antifungal was given clear zone as positive result of inhibition on *C. albicans*. RB.NC.EA exhibit widest clear zone than ketoconazole for *C. albicans* on concentration 10,000 µg/mL.

### Separation and TLC-bioautography

The crude extracts were separated through chromatography column with chloroform-methanol in gradual concentration. The positive result on TLC-bioautography was shown on fractionation CM91. The positive R<sub>f</sub> on RB.NC.EA\_CM91 were on 0.8625, 0.7625, and 0.6625. The significant clear zone was shown on R<sub>f</sub> 0.7625 (Figure 2). For NB.NC.EA, the R<sub>f</sub> was on 0.7125 (picture not shown). The fractionation CM91 of extract RB.NC.EA and NB.NC.EA were further separated with mobile phase CE55.

### Antimicrobial activity on fraction

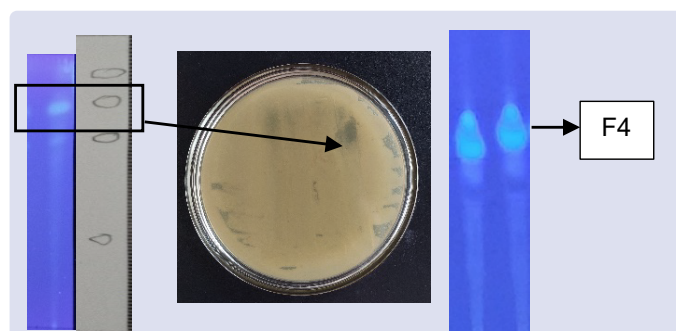
Fraction of RM.NC.EA (F4) able to inhibit *C. albicans* and bacteria. For NB.NC.EA (F5), it also able to inhibit *C. albicans* and bacteria, except *S. aureus* ATCC 6583. Both of fractions have inhibition concentration in the range 1,000 – 250 µg/mL. F4 and F5 has shown lower inhibition concentration than the crude extract, except *S. aureus* ATCC 6583 on F5. Generally, F4 and F5 have better inhibition on gram-negative bacteria than positive. While on crude extract the RB.NC.EA was shown wide diameter clear zone on *P. aeruginosa* and *C. albicans*, the fraction (F4) was giving similar range diameter (2.00 – 4.00 mm) to all pathogenic microbe tested.

### Identification of fractions

The result on identification exhibits the fractions were not single compound. The result on identification by LCMS/MS were describe on table 4, the structure of identified compound was on figure 3, and chromatogram was on figure 4. F1 and F4 further separation were conduct by TLC-separation with chloroform-ethyl acetate (5:5) and gave cumulation on time retention 9 - 10 minutes. For F2 and F3, without further separation, the peak was also detected on time retention 5 and 8 minutes. The mass spectrophotometry was using ion H<sup>+</sup> and Na<sup>+</sup> as ion adduct.

### Futoamide (1)

Futoamide was detected on F1 and F4. Futoamide given protonated molecular ion at m/z [M + ion adduct] 324.1569, ion <sup>+</sup>Na was used as adduct ion, the neutral mass 301.16779 for which molecular formula C<sub>18</sub>H<sub>23</sub>NO<sub>3</sub> was generated. The compound was tentatively identified as futoamide. The fragmentation on dioxole ring of this compound resulted in the presence of ions at m/z 272.16415 [M- CHO]<sup>+</sup>, and 122.05819 [M- C<sub>10</sub>H<sub>12</sub>-H<sub>2</sub>O-CHO]<sup>+</sup>. The fragmentation on aliphatic resulted in the presence of ion at m/z 149.05952 [M- C<sub>3</sub>H<sub>5</sub>-CNC(O)-C<sub>4</sub>H<sub>5</sub>]<sup>+</sup>.

**Figure 2. TLC-Bioautography of extract RM.NC.EA with CM91 as mobile phase on *C. albicans* (A and B) further separation with CE55 develop F4 (C).**

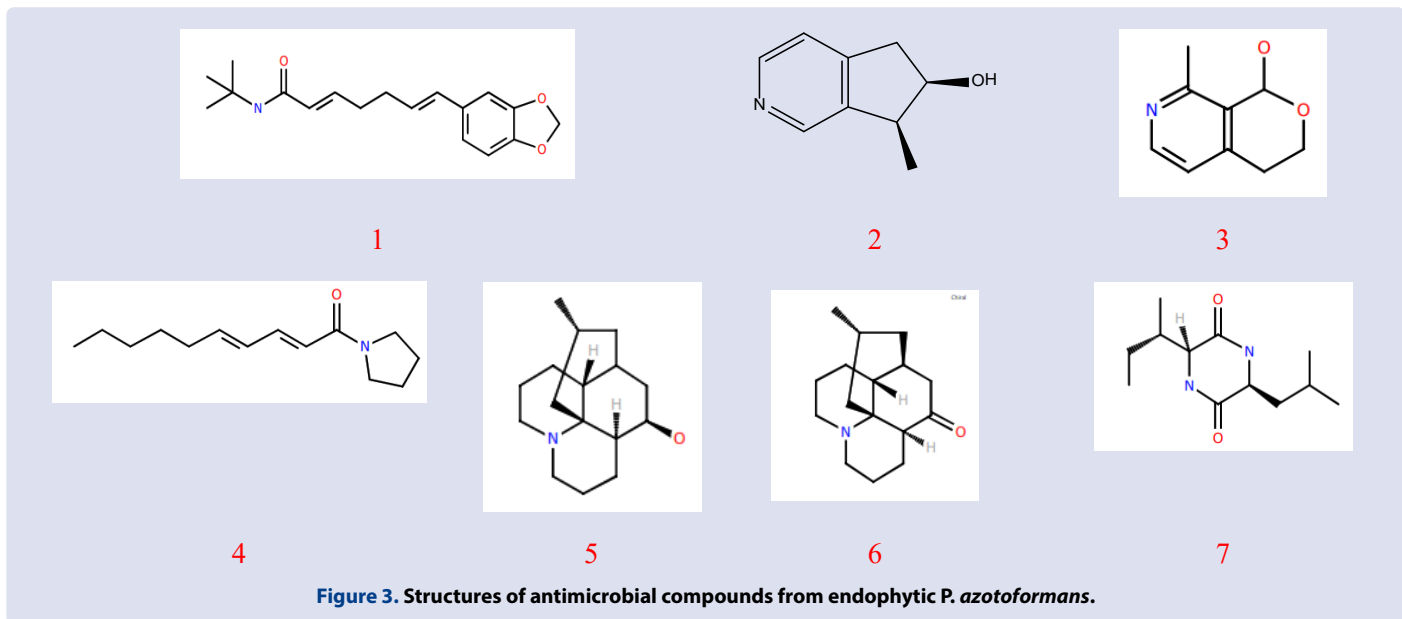


Figure 3. Structures of antimicrobial compounds from endophytic *P. azotoformans*.

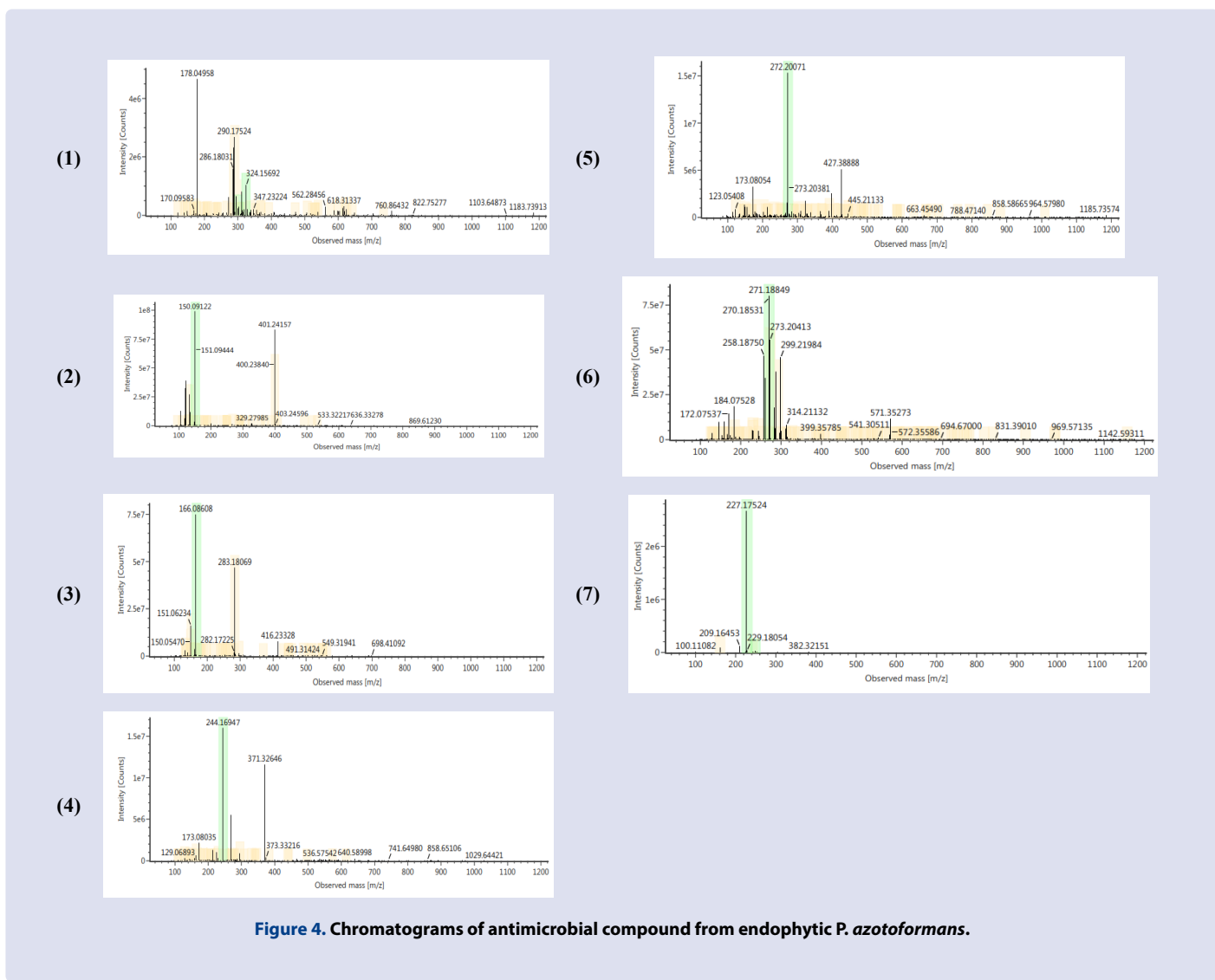


Figure 4. Chromatograms of antimicrobial compound from endophytic *P. azotoformans*.

**Table 2.** *In vitro* antibacterial activity on ethyl acetate extract of *P. azotoformans* using agar diffusion method.

| Conc..<br>(µg/mL) | Microorganism                 |                                |                                     |                             |                                    |                                |                                  |                                  |
|-------------------|-------------------------------|--------------------------------|-------------------------------------|-----------------------------|------------------------------------|--------------------------------|----------------------------------|----------------------------------|
|                   | <i>S. aureus</i><br>ATCC 6583 | <i>S. aureus</i><br>ATCC 25923 | <i>S. typhimurium</i><br>ATCC 25241 | <i>E. coli</i><br>ATCC 8739 | <i>P. aeruginosa</i><br>ATCC 15442 | <i>B. cereus</i><br>ATCC 10876 | <i>B. subtilis</i><br>ATCC 19659 | <i>C. albicans</i><br>ATCC 10231 |
| NB.NC.EA          |                               |                                |                                     |                             |                                    |                                |                                  |                                  |
| 50,000            | 3.33 ± 0.56                   | 2.50 ± 0.71                    | 2.00 ± 0.00                         | 1.33 ± 0.58                 | 2.00 ± 0.50                        | 2.00 ± 0.00                    | 2.50 ± 0.71                      | 5.50 ± 1.50                      |
| 10,000            | 1.50 ± 0.00                   | 1.00 ± 0.00                    | 2.00 ± 0.00                         | 2.33 ± 0.58                 | 2.00 ± 0.00                        | 2.00 ± 0.00                    | 1.00 ± 0.00                      | 3.00 ± 0.71                      |
| 5,000             | -                             | -                              | 2.00 ± 0.00                         | 1.83 ± 0.76                 | -                                  | 1.00 ± 0.00                    | -                                | 1.50 ± 0.50                      |
| 1,000             | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 100               | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| RM.NC.EA          |                               |                                |                                     |                             |                                    |                                |                                  |                                  |
| 50,000            | 4.00 ± 0.00                   | 4.50 ± 0.71                    | 4.50 ± 1.00                         | 3.50 ± 0.71                 | 14.50 ± 0.12                       | 3.00 ± 1.00                    | 3.00 ± 0.00                      | 13.25 ± 0.35                     |
| 10,000            | 4.00 ± 0.00                   | 3.00 ± 0.00                    | 4.00 ± 0.00                         | 5.67 ± 1.76                 | 4.00 ± 0.00                        | 1.00 ± 0.00                    | 1.50 ± 0.71                      | 11.50 ± 0.71                     |
| 5,000             | 1.50 ± 0.00                   | -                              | 4.00 ± 0.00                         | 3.50 ± 0.87                 | 3.75 ± 0.35                        | 6.00 ± 0.00                    | 2.75 ± 1.06                      | 8.50 ± 0.87                      |
| 1,000             | -                             | -                              | 2.00 ± 0.00                         | -                           | -                                  | -                              | 1.00 ± 0.00                      | 1.00 ± 0.00                      |
| 100               | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| Control           |                               |                                |                                     |                             |                                    |                                |                                  |                                  |
| C +               | 10.00 ± 1.41                  | 8.50 ± 0.00                    | 8.75 ± 0.35                         | 4.50 ± 0.00                 | 9.00 ± 0.00                        | 4.50 ± 0.70                    | 11.00 ± 0.71                     | 4.50 ± 1.41                      |
| C -               | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |

Result: Diameter of clear zone ± standard deviation (mm)

**Table 3.** *In vitro* antimicrobial activity on fraction of *P. azotoformans* using agar diffusion method.

| Cons.<br>(µg/mL)     | Microorganism                 |                                |                                     |                             |                                    |                                |                                  |                                  |
|----------------------|-------------------------------|--------------------------------|-------------------------------------|-----------------------------|------------------------------------|--------------------------------|----------------------------------|----------------------------------|
|                      | <i>S. aureus</i><br>ATCC 6583 | <i>S. aureus</i><br>ATCC 25923 | <i>S. typhimurium</i><br>ATCC 25241 | <i>E. coli</i><br>ATCC 8739 | <i>P. aeruginosa</i><br>ATCC 15442 | <i>B. cereus</i><br>ATCC 10876 | <i>B. subtilis</i><br>ATCC 19659 | <i>C. albicans</i><br>ATCC 10231 |
| NB.NC.EA_Zone 4 (F5) |                               |                                |                                     |                             |                                    |                                |                                  |                                  |
| 1,000                | -                             | 1.00 ± 0.00                    | 3.50 ± 0.00                         | 3.00 ± 0.00                 | 4.00 ± 0.00                        | 3.00 ± 0.00                    | 1.50 ± 0.00                      | 4.00 ± 0.00                      |
| 500                  | -                             | 1.00 ± 0.00                    | 1.00 ± 0.00                         | 2.33 ± 0.71                 | 1.50 ± 0.00                        | 2.00 ± 0.00                    | -                                | 1.00 ± 0.00                      |
| 250                  | -                             | -                              | 1.00 ± 0.00                         | 1.00 ± 0.00                 | -                                  | -                              | -                                | -                                |
| 125                  | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 62.5                 | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 31.25                | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 15.625               | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| RM.NC.EA_Zone 3 (F4) |                               |                                |                                     |                             |                                    |                                |                                  |                                  |
| 1,000                | 4.00 ± 0.00                   | 4.00 ± 0.00                    | 4.00 ± 0.00                         | 3.00 ± 0.00                 | 3.00 ± 0.00                        | 4.00 ± 0.00                    | 4.00 ± 0.00                      | 3.00 ± 0.00                      |
| 500                  | 3.00 ± 0.00                   | -                              | 3.00 ± 0.00                         | 2.50 ± 0.00                 | 2.50 ± 0.00                        | 3.00 ± 0.00                    | 3.00 ± 0.00                      | 2.00 ± 0.00                      |
| 250                  | -                             | -                              | 2.00 ± 0.00                         | 2.00 ± 0.00                 | -                                  | -                              | -                                | -                                |
| 125                  | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 62.5                 | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 31.25                | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| 15.625               | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |
| Control              |                               |                                |                                     |                             |                                    |                                |                                  |                                  |
| C +                  | 8.50 ± 0.00                   | 6.50 ± 0.00                    | 5.50 ± 0.00                         | 5.00 ± 0.00                 | 7.50 ± 0.00                        | 4.50 ± 0.00                    | 4.50 ± 0.00                      | 4.50 ± 0.71                      |
| C -                  | -                             | -                              | -                                   | -                           | -                                  | -                              | -                                | -                                |

## Gentialutine (2)

The compounds identified on F2 were Gentialutine (alkaloid) and Gentiaticetine. Gentialutine or (6R,7S)-7-methyl-6,7-dihydro-5H-cyclopenta[c]pyridin-6-ol forming protonated molecular ion [M+H]<sup>+</sup> at m/z 150.0912 with formula C<sub>9</sub>H<sub>11</sub>NO. The fragmentation 135.06681 [M- CH<sub>2</sub>]<sup>++</sup> was obtained from the fragmentation on methyl group; 120.08049 [M- CHO]<sup>++</sup> was obtained from the cleavage on cyclo-pentane ring which led the loss on one carbon bond to oxygen; further loss on carbon led the formation of ion 107.07233 [M- C<sub>2</sub>H<sub>2</sub>O]<sup>++</sup> and 106.06481 [M- CH<sub>2</sub>-CHO]<sup>++</sup>; further loss on another carbon led the formation of ion 94.06481 [M- C<sub>2</sub>H<sub>2</sub>-COH]<sup>++</sup> and 92.05121 [M- C<sub>3</sub>H<sub>5</sub>O]<sup>++</sup>.

## Gentiaticetine (3)

Gentiaticetine with formula C<sub>9</sub>H<sub>11</sub>NO<sub>2</sub> detected on peak ion m/z 165.07898. The fragmentation on the methyl group develops an ion

m/z 151.06236 [M- CH<sub>2</sub>]<sup>++</sup> and 150.05470 [M- CH<sub>3</sub>]<sup>++</sup>; fragmentation on alcohol group develops ion m/z 148.07529 [M- OH]<sup>++</sup>; further fragmentation with one carbon develop ion m/z 136.07425 [M- CHO]<sup>++</sup> and 134.05973 [M- CH<sub>2</sub>-OH]<sup>++</sup>; ion m/z 123.06763 [M- C<sub>2</sub>H<sub>2</sub>O]<sup>++</sup>; fragmentation on pyran ring develop ion m/z 122.09581 [M- CO<sub>2</sub>]<sup>++</sup> with the rearrangement of hydrogen and 120.08035 [M- CHO]<sup>++</sup> the formation of carbocyclic acid; further fragmentation develop ion m/z 107.07164 [M-CH<sub>2</sub>-CO<sub>2</sub>]<sup>++</sup> and 106.06473 [M- CH<sub>2</sub>-CHO]<sup>++</sup>.

## 1-[(2E,4E)-2,4-Decadienoyl]pyrrolidine (4)

1-[(2E,4E)-2,4-Decadienoyl]pyrrolidine was detected on peak ion m/z 221.17796 and the formula C<sub>14</sub>H<sub>23</sub>NO. The fragmentation on methyl group led the development ion m/z 208.16921 [M- CH]<sup>++</sup>; the fragmentation on pyrrole ring led the development ion m/z 137.09520 [M- C<sub>5</sub>H<sub>10</sub>N]<sup>++</sup> - the characteristic of N-methylpyrrolidines; ion m/z 123.07973 [M- CH<sub>2</sub>-C<sub>5</sub>H<sub>10</sub>N]<sup>++</sup>; and 109.06395 [M- C<sub>2</sub>H<sub>4</sub>-C<sub>5</sub>H<sub>10</sub>N]<sup>++</sup>.

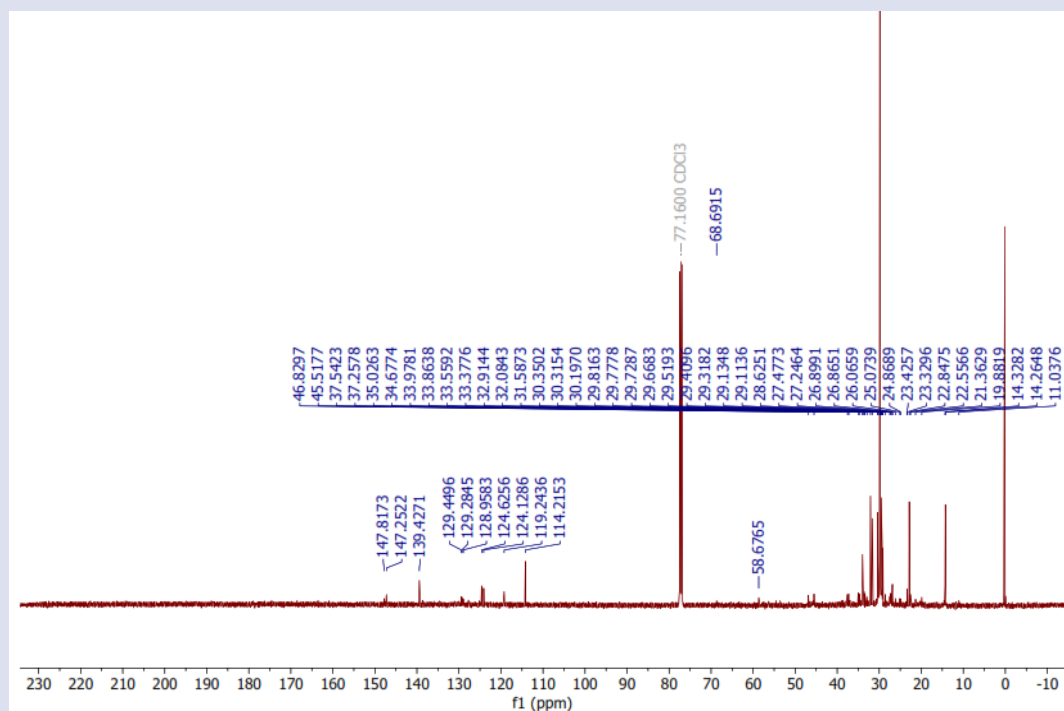


Figure 5. Spectral  $^{13}\text{C}$ -NMR of antimicrobial compound from endophytic *P. azotoformans*.

Table 4. Bioactive compound from fractions of *P. azotoformans* identified by LC-MS/MS.

| Fraction | $t_r$ | $m/z$<br>[M+adduct] | Adducts | Neutral mass | Mass error (mDa) | Fragmentation ions (observed peak)  | Formula  | Compound                                | Structure number |
|----------|-------|---------------------|---------|--------------|------------------|---|--|---|------------------|
| F1       | 10.34 | 324.1569            | +Na     | 301.16779    | -0.1             | 290.17548; 288.19585; 286.18009; 272.16415; 149.05952; 122.05819  | $\text{C}_{18}\text{H}_{23}\text{NO}_3$          | Futoamide                               | 1                |
|          | 10.21 | 260.1643            | +H      | 259.15723    | -0.2             | 174.0547; 158.0597; 120.0439  | $\text{C}_{16}\text{H}_{21}\text{NO}_2$          | Candidate Mass 1                        |                  |
|          | 10.24 | 276.1596            | +H      | 275.15214    | 0.1              | 174.0547; 158.0597; 120.0439  | $\text{C}_{16}\text{H}_{21}\text{NO}_3$          | Candidate Mass 2                        |                  |
| F2       | 9.79  | 150.0912            | +H      | 149.08406    | -0.1             | 135.06681; 134.09621; 133.08812; 120.08049; 107.07233; 106.06483; 94.06481; 92.05121                                    | $\text{C}_9\text{H}_{11}\text{NO}$               | Gentialutine                            | 2                |
|          | 9.13  | 166.0861            | +H      | 165.07898    | -0.2             | 151.06236; 150.05470; 149.04724; 148.07529; 136.07425; 134.05973; 123.06763; 122.09581; 120.08035; 107.07164; 106.06473 | $\text{C}_9\text{H}_{11}\text{NO}_2$             | Gentiatibetine                          | 3                |
|          | 9.88  | 400.2384            | +H      | 399.23106    | 0.1              | 253.16971; 134.09621  | $\text{C}_{26}\text{H}_{29}\text{N}_3\text{O}$   | Candidate Mass 3                        |                  |
| F3       | 9.03  | 283.1807            | +H      | 282.17321    | 0.2              | 163.09435; 162.09116; 134.09596   | $\text{C}_{18}\text{H}_{22}\text{N}_2\text{O}$   | Candidate Mass 4                        |                  |
|          | 10.31 | 311.1755            | +H      | 310.16813    | 0.1              | 296.29501; 176.07041; 162.09117; 148.07541  | $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}_2$ | Candidate Mass 5                        |                  |
|          | 9.98  | 244.1693            | +Na     | 221.17796    | 2.2              | 159.06751; 146.05960  | $\text{C}_{14}\text{H}_{23}\text{NO}$            | 1-[(2E,4E)-2,4-Decadienoyl] pyrrolidine | 4                |
|          | 10.40 | 272.2006            | +Na     | 249.20926    | 2.1              | 216.17409   | $\text{C}_{16}\text{H}_{27}\text{NO}$            | Dihydrolycopodine                       | 5                |
|          | 10.31 | 270.1853            | +Na     | 247.19361    | 2.5              | 232.16907; 232.16885; 218.15439   | $\text{C}_{16}\text{H}_{25}\text{NO}$            | Lycopodine                              | 6                |
|          | 10.58 | 300.2319            | +H      | 299.22491    | -0.3             | 298.21648; 198.09089; 184.07522; 172.07544; 159.06758; 130.06467  | $\text{C}_{20}\text{H}_{29}\text{NO}$            | Candidate Mass 6                        |                  |
|          | 10.64 | 326.2476            | +H      | 325.24056    | -0.3             | 184.07522; 298.216.48; 184.07522; 172.07544; 159.06758; 146.05974   | $\text{C}_{22}\text{H}_{31}\text{NO}$            | Candidate Mass 7                        |                  |
| F4       | 10.37 | 324.1568            | +Na; +H | 301.16779    | -0.2             | 290.17392; 272.16476; 149.05938; 122.05918  | $\text{C}_{18}\text{H}_{23}\text{NO}_3$          | Futoamida                               | 1                |
|          | 10.24 | 270.1852            | +Na     | 247.19361    | 2.4              | 232.16877; 192.13820  | $\text{C}_{16}\text{H}_{25}\text{NO}$            | Lycopodine                              | 6                |
|          | 10.27 | 276.1594            | +H      | 275.15214    | 0.0              | 260.18519; 184.07529; 178.0497  | $\text{C}_{16}\text{H}_{21}\text{NO}_3$          | Candidate Mass 8                        |                  |
|          | 10.42 | 304.1905            | +H      | 303.18344    | -0.2             | 298.21636; 286.18024; 184.07508; 160.03849; 107.08456   | $\text{C}_{18}\text{H}_{25}\text{NO}_3$          | Candidate Mass 9                        |                  |
|          | 10.51 | 324.2316            | +H      | 323.22491    | -0.5             | 254.16918; 198.09032; 98.98333  | $\text{C}_{22}\text{H}_{29}\text{NO}$            | Candidate Mass 10                       |                  |
| F5       | 10.09 | 244.1695            | +Na     | 221.17796    | 2.3              | 159.06742; 130.06480  | $\text{C}_{14}\text{H}_{23}\text{NO}$            | 1-[(2E,4E)-2,4-Decadienoyl] pyrrolidine | 4                |
|          | 5.43  | 227.1752            | +H      | 226.16813    | -0.2             | 209.16420; 100.11142  | $\text{C}_{12}\text{H}_{22}\text{N}_2\text{O}_2$ | Cyclo-(Leu-Ile)                         | 7                |
|          | 10.27 | 272.2007            | +Na     | 249.20926    | 2.2              | 159.06748; 149.02288  | $\text{C}_{16}\text{H}_{27}\text{NO}$            | Dihydrolycopodine                       | 5                |
|          | 10.35 | 322.0797            | +Na     | 299.09061    | -0.2             | 236.97797; 225.07253  | $\text{C}_{15}\text{H}_{13}\text{N}_3\text{O}_4$ | Candidate Mass 11                       |                  |
|          | 10.48 | 457.2766            | +Na     | 434.28797    | -0.5             | 317.19561; 155.06975  | $\text{C}_{22}\text{H}_{42}\text{O}_8$           | Candidate Mass 12                       |                  |

## Dihydrolycopodine (5) and Lycopodine (6)

Dihydro-lycopodine was detected at ion  $m/z$  249.2096 with formula  $C_{16}H_{27}NO$ . The fragmentations on alcohol group and methyl group led the ion  $m/z$  216.17409  $[M - CH_3 - H_2O]^+$ . Lycopodine was detected at ion  $m/z$  247.19361 with formula  $C_{16}H_{25}NO$ . The fragmentation on methyl group led the ion  $m/z$  232.16907  $[M - CH_3]^+$ ; further fragmentation led development ion  $m/z$  218.15439  $[M - C_2H_5]^+$ , and 192.13829  $[M - C_4H_7]^+$ .

The  $^{13}C$ -NMR on F1 with mixture compound condition (Figure 5) was exhibit aromatic signal (ppm) at  $\delta C$  147.25 (C) and 147.25 (C). Signal between 110-130 (CH) ppm might be aromatic signal or alkenes. Signal at 58.68 (C) ppm come from alkanes which bound with nitrogen, oxygen or halogen. The intense peak on the range at 30 ppm revealed the aliphatic carbon of alkanes as CH<sub>2</sub>. Signal on area 5-45 ppm also might be carbon sp<sup>3</sup>. The  $^{13}C$ -NMR revealed the structure of compound (1) (Figure 6) with mixture of aliphatic carbon. The mixture of aliphatic carbon might come from fatty acid chain. However, there's no identified or detected signal on 170 ppm, hence the aliphatic carbon might come from mixture compounds.

## DISCUSSION

The crude extract was more effective to inhibit *C. albicans* as yeast rather than bacteria. The antimicrobial activity differences might be related to the cell wall composition of microorganism and the antimicrobial compound itself. The major components of the *C. albicans* cell wall are fibrillar polysaccharides and protein.<sup>14</sup> For bacteria like *S. aureus*, the major components of the cell wall are murein teichoic acid and protein. Murein consists of glycan strands, which are cross-linked by peptide bridges.<sup>15</sup>

The fractions able to go lower on inhibition concentration than the crude extract. However, the diameter clear zone resulted from fractions were in the range 1.00 – 4.00 mm, which is considered low inhibition.

F1 containing compound futoamide (1), candidate mass 1 and candidate mass 2. F1 has shown antimicrobial activity on the pathogenic microbes-on previous research.<sup>4</sup> Futoamide (1) containing benzene ring

attached with dioxol (benzodioxole), heptadiene as polyunsaturated fatty acid, and isobutyl amide. Grouped as alkaloamide or isobutyl amide alkaloid syntheses from L-lysine, futoamide was also found on family *Piperaceae*, with bioactive activity as antioxidant. Futoamide in *Piper* sp. is homolog to pellitorine, an insecticidal, antimycobacterial, anticandidal, and anti-plasmodial compound.<sup>16,17</sup> Pellitorine was also found in bioactive compound of *Pseudomonas stutzeri* which act to inhibit *Mycobacterium*. Beside pellitorine, *P. stutzeri* also able to produce brachystamide B, guineensine, piperlonguminine, and retrofractamide, bioactive compound with benzodioxol group attached to unsaturated alkyl amide.<sup>18</sup> The bioactive properties on futoamide lay on its alkyl amide structure and on its benzodioxole group. Benzodioxole as oxygen heterocyclic compound is an antibacterial and antifungal compound.<sup>19</sup> Candidate mass 1 and 2 based on ion  $m/z$  peaks are 2-heptil-3-hydroxyquinolone and 3-n-heptil-3-hydroxy-1,2,3,4-tetrahydroquinoline-2,4-dione, respectively. Alkyl-hydroxyquinolines frequently found on *Pseudomonas* spp. as quorum-sensing molecule. The compounds also have been identified on marine-derived *Streptomyces* sp., it has strong anticandidal than antibacterial effect. It has an ability to inhibit hyphal growth of *C. albicans*.<sup>20</sup> F1 also has strong anticandidal activity than its antibacterial activity.<sup>4</sup> Therefore, this identification compound supported that F1 has a potential as anticandidal compound.

F2 consist of compound gentialutine (2), gentiatibetine (3), candidate mass 3, candidate mass 4 and candidate mass 5. Gentialutine (2) and gentiatibetine (3) are monoterpene alkaloid<sup>13</sup> containing pyridine nucleus, synthesis from nicotinic acid. Pyridine ring has an ability as antibacterial activity. The potential lays on a proton-accepting nitrogen atom (heterocyclic or non-heterocyclic) and one or more hydrogen proton donating hydrogen atom.<sup>21</sup> The pyridine ring on gentialutine fused with cyclo-pentane, and on gentiatibetine, it's fused with pyran ring. Gentialutine (2) first isolated from root of *Gentiana lutena* and leaf of *Menyanthes trifoliata*.<sup>22</sup> But, there wasn't any report to be produced by Pseudomonaceae, the similar compound of cyclo-pentane pyridine has been reported to be isolated from marine fungi (*Wallemia sebi*) and it has weak antibacterial against *Enterobacter*.<sup>23</sup> Thus, also reflected on our research where F2 only inhibit *S. aureus* ATCC 6583 on 1,000  $\mu g/mL$  and gave negative result for others microbes. Several

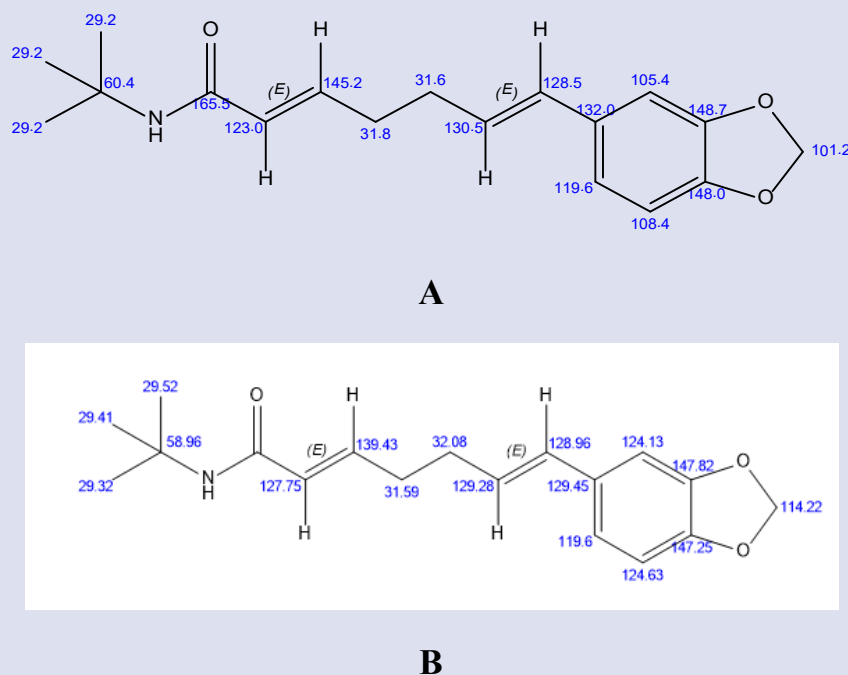


Figure 6. The structure of futoamide from endophytic *P. azotoformans*: prediction (A) and based on result (B).

report on gentiatabentine (3) was identified on ethyl acetate extracted from *Streptomyces* with ability against *Plasmodium* parasite<sup>24</sup> and from methanol extract of *Melophlus sarasinorum* (sponge marine) with ability as antioxidant.<sup>25</sup> But, there hasn't any report on gentiatabentine antimicrobial activity, pyranopyridines as an antibacterial compound has been reported with mechanism interrupting the efflux pump on *E. coli*.<sup>26</sup>

F3 have several compounds like 1-[(2E,4E)-2,4-Decadienoyl]pyrrolidine (4), dihydro-lycopodine (5), lycopodine (6), candidate mass 6, and candidate mass 7. The 1-[(2E,4E)-2,4-Decadienoyl]pyrrolidine (4) is an alkaloid compound, containing pyrrolidine nucleus synthesis from L-ornithine, and unsaturated fatty acid.<sup>13,17</sup> It also known as sarmentine, mostly found in *Piper* sp.<sup>13,16</sup> It has potency as herbicide<sup>16</sup> and antimicrobial compound.<sup>27</sup> Based on research methanol extract of *Piper sarmentosum* that contain sarmentine, it was able to inhibit *Mycobacterium tuberculosis*, Methicillin Resistant *Staphylococcus aureus* (MRSA), *S. aureus*, *E. coli* and *P. aeruginosa*.<sup>18,27</sup> From our previous research, F3 also have antibacterial and anticandidal activity.<sup>4</sup> The interesting study was reported on cytotoxic activity of sarmentine, but indeed its activity was still lower than taxol as positive control.<sup>17</sup> From our observation, there hasn't any publication on sarmentine from Pseudomonaceae. The similar ring of pyrrolidine compound was isolated from *Pseudomonas* sp. as N-(decanoyl)pyrrolidine and N-(decenoyl)pyrrolidine with antimicrobial activity against several microbe.<sup>28</sup>

Dihydrolycopodine (5) and lycopodine (6) grouped as Lycopodium-alkaloid, it was first isolated on moses Lycopodiaceae.<sup>29,30</sup> The multicyclic of lycopodine biosynthesis arise from acetoacetyl co-A and L-lysine. The lycopodium alkaloid has been well explained regarding the source, classes, and potency. It's also mentioned the characteristic of lycopodium alkaloid on acetylcholinesterase inhibition activity.<sup>30</sup> As for its bioactivity, lycopodine and dihydrolycopodine from *Lycopodium clavatum* extracted with chloroform-alcohol has been discovered to have antioxidant, antiviral, and antimicrobial activity against *E. coli*, *P. aeruginosa*, *Proteus mirabilis*, *Acinobacter baumannii*, *Klebsiella pneumoniae*, *S. aureus*, *B. subtilis*, *C. albicans*, and *C. parapsilosis*.<sup>31</sup> Thus, strengthen our result from previous research on F3, on its ability as anticandidal. Lycopodine has been discovered on endophytic fungus *Paraborenia* from *Lycopodium serratum*<sup>32</sup>, but the bioactive activities has not been explored, yet. The candidate compound 6 based on ion m/z fragmentation is 2-decyl-1-methyl-quinolin-4-one. It was also reported as antimicrobial compound from endophytic marine *Pseudomonas* sp.<sup>33</sup>

F4 consist of futoamida (1), lycopodine (6), candidate mass 8, candidate mass 9, and candidate mass 10. There hasn't any single publication on lycopodine isolated from Pseudomonaceae. However, pelletierine as lycopodine intermediat was also found on *P. azotoformans*.<sup>34</sup> Therefore, further studies were needed to support this finding.

F5 consist of compound (4), cyclo-(leu-ile) (7), dihydro-lycopodine (5), candidate mass 11, and candidate mass 12. The cyclo-(leu-ile) (7) or 3-butan-2-yl-6-(2-methylpropyl)piperazine-2,5-dione is a diketopiperazine, grouped as cyclo-dipeptide (CDP) alkaloid. CDP can be produced by bacteria, fungi and plants. CDP synthesis by enzyme NRPS. CDP has bioactivities as antioxidant, antiviral, antitumor, antifungal and antibacterial<sup>18,35,36</sup>, and plant growth regulation.<sup>37</sup> The reported CDP from *Pseudomonas* were cyclo-(leu-pro), cyclo-(val-pro), cyclo-(pro-tyr), and cyclo-(meth-pro).<sup>18</sup> Some CDP in *Pseudomonas* are involved in quorum sensing that mediated plant growth.<sup>36</sup> Cyclo-(leu-ile) has been reported to be identified on marine *Bacillus subtilis*<sup>38</sup>, and chiral as cyclo-(ile-leu) was identified on fungus *Verruculina enalia*<sup>39</sup> but its bioactivity has not been explored, yet. From our exploration, there's no cyclo-(leu-ile) has been reported to be isolated from *Pseudomonas*. CDP has potential as antimicrobial, for instance cyclo-(arg-pro) from marine *Pseudomonas* sp. that has been developed as antibiotic.<sup>40</sup>

Alkaloids on the microorganism play a role as inhibitors compound for competitors, thus functionally as antimicrobial compound. Alkaloid soluble on non-polar solvent like chloroform and also soluble on alcohol.<sup>21</sup> Alkaloid soluble on water at acidic condition and soluble on lipid at neutral and basic condition. Thus characteristic, give ability on alkaloid to pass membrane in the neutral form and ability to be transported in protonated form. Several alkaloid containing nitrogen and/or oxygen heterocyclic that rich with electron therefore it can act as proton acceptor and donor, and forming interaction like hydrogen bond, dipole-dipole interaction, van der Waals, and  $\pi$ -stacking interaction with biomolecular target like enzyme and receptor.<sup>8</sup> Thus ability explaining the antimicrobial mechanism on alkaloid, rather it is specific interaction with specific biomolecular target (ligand binding) or non-specific interaction (electrostatic and hydrophobic interaction).<sup>41,42</sup>

Antimicrobial mechanism of alkaloid like inhibition of sortase, inhibition of biofilm formation and inhibition of secretion system. Alkaloid able to interrupt protein synthase of cell wall thus lead the rupture on the cell wall and disrupt the turgor of cytoplasm. Alkaloid with low molecular weight might be able to diffuse into cell, and attenuated the influx-efflux mechanism in cytoplasm that led the cell lysis. But if the cell able to increase the cell efflux to pump out the alkaloid, the cell will be proceed to maintain the cell integrity.<sup>9,21</sup> F4 and F5 has better inhibition on *S. typhimurium* and *E. coli*, than any others bacteria. Aside efflux pump, alkaloid like cyclo-dipeptide might be attacking pore on the membrane cell bacteria by cationic-anionic affinity of amino group. Several alkaloid like quinolone have anti-quorum sensing to others bacteria and attenuated electron transport mechanism.<sup>21,37</sup> Alkaloid mechanism of action on *C. albicans* were due to inhibition of filament formation and the disruption of biofilm formation that prevent the adhesion of *C. albicans* to target (anti-adhesin), and *C. albicans* cell wall components. Alkaloid with aliphatic chain able to diffuse into *C. albicans* cell.<sup>41,42</sup>

From identification of crude extract and the fractions, synergism effect might apply on the antimicrobial compound of endophyte *P. azotoformans*. Synergism effect were found on different class of natural product. Anticandidal natural product might have synergism effect<sup>41</sup>, as several antimicrobial which combine the antimicrobial mechanism to strengthen the inhibition or to prevent increasing risk infection by others pathogen.<sup>42</sup>

Regarding from the publication on *P. azotoformans* gene cluster on secondary metabolite that consist of 4 NRPS gene cluster, 3 bacteriocin gene cluster, 1 terpen gene cluster and 1 unspecified gene cluster,<sup>43</sup> thus reflected on the antimicrobial compound identified in this research. The expression of secondary metabolite might be influenced by environment factors. This result describes the extraction and elucidates the identified antimicrobial compound of *P. azotoformans*, which can be further explored for the potency on medicinal and industrial area.

## CONCLUSION

*P. azotoformans* strain UICC B-91 has potency as antimicrobial compound with range of minimum inhibition was 1,000 - 125  $\mu$ g/mL. Anticandidal activities on *P. azotoformans* extracts were on synergism effect. Antimicrobial compounds have been discovered from *P. azotoformans* UICC B-91 as alkaloid on CHON arrangements with molecular weight approximately 100-450 Da. The identified alkaloid group are isobutyl amide alkaloid, monoterpene alkaloid, lycopodium alkaloid and cyclo-dipeptide alkaloid.

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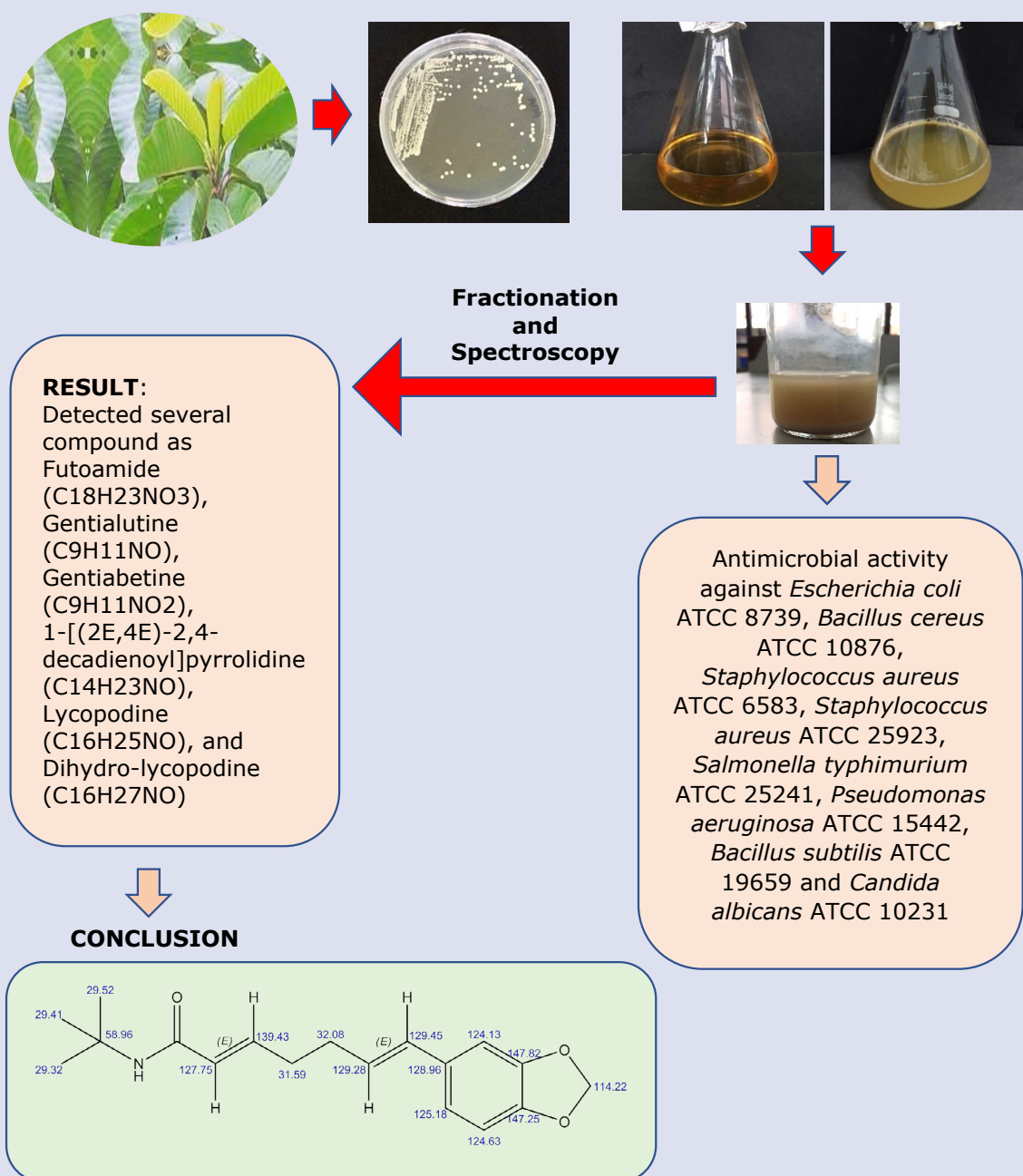
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## GRAPHICAL ABSTRACT



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