Entomopathogenic fungi *Beauveria* sp. and *Aspergillus sclerotiorum* can produce secondary metabolite quinidine

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Abstract. Entomopathogenic fungi have been reported to produce secondary metabolite quinolines. One of the quinoline alkaloids is quinidine, a compound that has antiarrhythmic properties, therefore it has an economic important value. The aim of the experiment reported here was to study the potency of entomopathogenic fungi *Beauveria* sp. IPBCC.19.1499 and *Aspergillus sclerotiorum* IPBCC.19.1500 in producing quinidine. The quinidine was obtained from a broth culture of each fungus grown in Potato Dextrose Broth medium (pH 6.2, static, room temperature) for 7, 14, and 21 days incubation period. At harvest, broth of each culture was extracted using chloroform. The extract then dried using an evaporator. The presence of quinidine in the extracted was detected by HPLC. The dry weight of fungal biomass and the crude extract of broth culture were also recorded. HPLC analysis showed that quinidine was produced by both fungi tested. The species of the fungus and incubation period significantly affected quinidine production. *Beauveria* sp. IPBCC.19.1499, in general, produced quinidine in higher concentration than that of *A. sclerotiorum* IPBCC.19.1500. The quinidine concentration increased significantly with increasing incubation period. The quinidine concentration was also positively correlated with biomass dry weight, while the crude extract dry weight was negatively correlated with quinidine concentration.

Keywords: Crude extract, HPLC, incubation period, secondary metabolic, static

1. Introduction

*Beauveria bassiana* [1] and *Aspergillus sclerotiorum* are known as entomopathogenic fungi [2]. Some entomopathogenic fungi produce various secondary metabolites, i.e. *B. bassiana* produces cyclodepsipeptide [3] and beauveriolide compounds [4, 5]. Cyclodepsipeptide compounds have the neuroprotective properties [6], while beauveriolide is a candidate for the prevention and treatment of nerve degeneration diseases such as Alzheimer's and Parkinson's disease [7, 8]. Penicillic acid produced by *A. sclerotiorum* can be used as biocontrol agent for *Phytophthora* disease [9].

Besides as entomopathogenic fungi, *B. bassiana* [10] and *Aspergillus* sp. [11] have been reported to be endophytic fungi. Some endophytic fungi of *Cinchona* plants such as *Diaporthe* sp. [12], *Arthrinium* sp., *Phomopsis* sp., *Schizophyllum* sp., *Penicillium* sp., *Fomitopsis* sp. [13], and *Fusarium* spp. [14], *Diaporthe* spp. [15], are known to produce quinoline alkaloid compounds. Quinoline alkaloid compounds include quinine, quinidine, cinchonine, and cinchonidin. Quinoline has a variety of benefits, can be used as antimalarials [16], antimicrobials [17], tailoring ingredients in soft drinks [18], pesticides [19], anticancer [20], antibacterial, antifungal, anthelmintic, anticonvulsant, ant-
inflammatory, and analgesic activity [21]. Quinidine is one of the quinoline alkaloids that can be used as antiarrhythmics [22].

Entomopathogenic *Penicillium* isolated from insect larvae (*Margaronia pyloalis* Welker) produced Quinolone (quinoline alkaloids) [23]. Yet, ability of entomopathogenic fungi such as *B. bassiana* and *A. sclerotiorum* in producing quinoline alkaloids, particularly quinidine has not been explored. Therefore, the research aimed to study ability of *Beauveria* sp. and *A. sclerotiorum* isolated from died insects in producing quinidine.

## 2. Materials and methods

### 2.1. Preparation of fungal culture

*Beauveria* sp. IPBCC.19.1499 and *A. sclerotiorum* IPBCC.19.1500 were grown separately on Potato Dextrose Agar media for 7 days after inoculation. The 7-days-old cultures were used as fungal inoculum.

### 2.2. Quinidine production and extraction

Three mycelial plugs (0.8 cm diameter) were taken from 7-days old fungal cultures of *Beauveria* sp. IPBCC.19.1499 and *A. sclerotiorum* IPBCC.19.1500 and were separately transferred into 50 ml Potato Dextrose Broth (PDB) media (pH: 6.2) in a 250 ml Erlenmeyer. The cultures were placed in a static condition at room temperature [14] for 7, 14, and 21 days of incubation. The experiment was done in three replicates.

At the end of each incubation period, the filtrates of each flask were separated from the mycelial biomass using sterile filter paper. The mycelia were dried in an oven at 60 °C until the mycelial dry weight was constant. The mycelial biomass was weighed and the dry biomass was recorded. The culture filtrate was mixed with chloroform (1:1 v/v) and was homogeneous for 10 minutes, then incubated for 24 hours at room temperature. The mixture was put into a separating funnel and left for a few minutes until two phases were formed. The lower phase was collected, then concentrated by a rotary evaporator at a temperature of 45 °C and a speed of 60 x g until a paste was formed. The pasta was weighed to obtain crude extract weight and stored at 4°C until used.

### 2.3. Quinidine detection using HPLC

The crude extract was dissolved in 1 mL of methanol and then as much as 20 µL of the extract was injected into HPLC. The HPLC conditions were as follows: Thermo C-18 (4.6 × 250 mm) column, mobile phase methanol: acetonitrile (80:20), the flow rate of 1.0 mL min⁻¹, temperature 40 °C, and detected at a wavelength of 210 nm [14]. The analysis was carried out qualitatively and quantitatively. Qualitatively, quinidine was determined based on its retention time (Rt), which is the same as standard quinidine. Quantitatively, quinidine was determined based on standard quinidine curve using several concentrations, vis 0, 1, 2, 3, 4, and 5 ppm. The standard curve with regression equation of $y = ax + b$ was used to calculate the quinidine content of the extract with the x-axis as the quinidine concentration and the y-axis as the quinidine peak area in a chromatogram.

### 2.4. Data analysis

Quinidine production, dry weight of crude extract and biomass dry weight were analyzed statistically using the SPSS 22.0 program. Data, which were significantly different were further tested using Duncan Multiple Range Test (DMRT) at the level of 5%. Relationships between the quinidine production, crude extract weight, and dry biomass were determined using the Pearson correlation.

## 3. Results

The results showed that *Beauveria* sp. IPBCC.19.1499 and *A. sclerotiorum* IPBCC.19.1500 produced quinidine. This was indicated by the presence of peaks that had similar retention times (Rt) with standard quinidine. A single quinidine peaks in standard quinidine appeared at Rt 1,892 ± 0.0125
minutes (figure 1), while in the extract of all fungi tested in all incubation periods showed more than one peak with different Rt (figure 2).

**Figure 1.** Quinidine standard chromatogram. (a) 1 ppm (b) 2 ppm. Remarks: the quinidine peak has shown by an arrow. The ordinate is absorbance (mAU) and axis is retention time (minutes).

The standard quinidine curve showed linearity \((y = 79777x - 4350)\) with correlation coefficients \(R^2\) of 0.9999. Based on the standard quinidine curve, *Beauveria* sp. IPBCC.19.1499 generally produces quinidine with a higher concentration than that of *A. sclerotiorum* IPBCC.19.1500 (table 1). Quinidine concentration production is influenced by the incubation time. The quinidine concentration in both fungi increased with the length of the incubation period. Statistical analysis showed that the quinidine concentration of *Beauveria* sp. IPBCC.19.1499 at 7-days incubation was 2.873 mg/L and 14-days incubation was 3.303 mg/L. The values were not significantly different from that of *A. sclerotiorum* IPBCC.19.1500 at 21-days incubation, which was 3.055 mg/L. *Beauveria* sp. IPBCC.19.1499 at 21-days incubation period, however, had the highest concentration (4.125 mg/L), and the value was significantly different compared to the quinidine concentrations produced at various incubation periods in both fungi tested (table 1). Conversely, the weight of crude extract of all incubation treatments tend to decrease in both fungi, but their weight was not significantly different.

The observation of fungal growth for 21-days incubation showed that the growth phase of the *Beauveria* sp. IPBCC.19.1499 differed from that of the growth phase of *A. sclerotiorum* IPBCC.19.1500 in terms of the increase in dry mycelium biomass. The growth of *Beauveria* sp. IPBCC.19.1499 was faster than *A. sclerotiorum* IPBCC.19.1500. The dry weight of *Beauveria* sp. IPBCC.19.1499 biomass in the first and second weeks increased but starting to decrease during the third-week incubation. In contrast, *A. sclerotiorum* IPBCC.19.1500 growth was slower than *Beauveria* sp. IPBCC.19.1499 at the same incubation time.

Pearson correlation analysis showed a positive correlation between the quinidine content and their dry biomass in both *Beauveria* sp. IPBCC.19.1499 and *A. sclerotiorum* IPBCC.19.1500 (table 2). The correlation between the quinidine content and dry biomass of *Beauveria* sp. IPBCC.19.1499 was weak \((r_p = 0.242)\), whereas *A. sclerotiorum* IPBCC.19.1500 was strong \((r_p = 0.999)\). In contrast, Pearson correlation of the quinidine content of crude extract to the crude extract weight (table 2) in both fungi showed \((r_p = -0.581; r_p = -0.787)\) a negative correlation, an increase in the quinidine amount in the crude extract was not followed by increasing in the crude extract. The Pearson correlation between *Beauveria* sp. IPBCC.19.1499 dry biomass and the crude extract weight showed a positive correlation. In *A. sclerotiorum* IPBCC.19.1500, however, a negative correlation between the dry biomass and the crude extract weight was found (table 3).

4. Discussion
Quinidine was frequently reported to be produced by endophytic fungi in *Cinchona* plants, such as *Diaporthe* spp. [15], *Schizophyllum* sp., *Penicillium* sp., *Phomopsis* sp., *Fomitopsis* sp., *Arthrinium* sp.
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[13], and Fusarium spp. [14]. These fungi also produce quinine, cinchonine, and cinchonidine [14, 13, 15]. This study found that quinidine was not only produced by fungal endophytes. Beauveria sp. IPBCC.19.1499 and A. sclerotiorum IPBCC.19.1500 are entomopathogenic fungi that are able to produce quinidine. This is the first report of entomopathogenic fungi in producing quinidine. An entomopathogenic fungi, B. bassiana ATCC 7159 was reported to produce quinolones [24], but not quinidine. This fungus reduced quinoline-3-carboxylic acid to 3-(hydroxymethyl) quinoline [24]. Quinoline is also found in non-entomopathogenic fungus, Trichoderma asperellum fungal extract. However, this fungus was stated as potentially used as a bio-insecticide for mosquito larvae [25].

Quinidine can be produced in a liquid medium by endophytic fungi within 1-21 days of the incubation period. Quinidine (2,873 mg/L and 1,275 mg/L) in Beauveria sp. IPBCC.19.1499 and A. sclerotiorum IPBCC.19.1500 fungi studied were detected at 7 days of incubation. Maehara et al. [13] showed that quinidine produced at 1-4 days incubation period with concentrations of less than 10 µg/L-50 µg/L. Shibuya et al. [26] detected quinidine from Diaporthe sp. as much as 3-5 µg/L under conditions of pH 5.6 for 10 days of incubation, while at pH 6.2 the production was as much as 2-5 µg/L. Quinine production could also be stimulated by adding bark powder of Cinchona succirubra in PDB culture medium. The quinine production increased to 2 fold than that of in the endophytic fungus culture grown in the control medium for 3 days [27].

Beauveria IPBCC.19.1499 and A. sclerotiorum IPBCC.19.1500 had different growth rates based on the dry biomass. Beauveria sp. IPBCC.19.1499 had a higher growth rate than A. sclerotiorum IPBCC.19.1500 at the 7 and 14 days of incubation, then dry biomass decrease at 21 days incubation, while A. sclerotium increased in all incubation treatments. Prosser [28] reported that the number of cells increment indicates that the growth of microorganisms. Fungal growth is influenced by environmental factors such as temperature, pH, and the state of the air atmosphere [29]. In general, the growth of fungi incubated for 21 days has entered the stationary phase [30], and in this phase, most fungi produce secondary metabolites [31, 32]. In previous reports, incubation of fungi was also carried out for 21 days [14, 15].

The increase in Beauveria IPBCC.19.1499 dry biomass that occurred in the first and second week was accompanied by an increase in the extract weight and quinidine concentration. Conversely, in the third week (incubation period of 21 days) a decrease in dry biomass was followed by a decrease in crude extract weight, even though all the weight of crude extract of all treatments was not significantly different, but the quinidine concentrations increased. At 21 days of incubation, Beauveria IPBCC.19.1499 is likely to be in a stationary phase, therefore, dry biomass decreases, and more secondary metabolites, including quinidine, were produced. The peaks that appeared on the chromatogram of the crude extract might be the secondary metabolites produced in the stationary phase. The tendentious decreasing the crude extract weight in Beauveria sp. IPBCC.19.1499 is probably caused by stress condition, which leads to high production of quinidine at 14 and 21 days incubation.

Dry biomass of A. sclerotiorum IPBCC.19.1500 increased with incubation time, but the weight of the crude extract decreased and the quinidine concentration increased. A. sclerotiorum IPBCC.19.1500 at 21 days incubation had not entered the stationary phase, therefore the dry biomass still increased compared to the incubation period at 7 and 14 days. In addition, secondary metabolites in A. sclerotiorum IPBCC.19.1500 already produced in exponential mycelial growth. The crude extract weight of A. sclerotiorum IPBCC.19.1500 tend to decrease in a longer period of incubation. This was probably, like in Beauveria sp IPBCC.19.1499, caused by stress condition which in constrast leads to high production of quinidine at 14 and 21 days incubation.

Quinidine was produced by Beauveria sp. IPBCC.19.1499 and A. sclerotiorum IPBCC.19.1500 in a static state incubation. Our preliminary studies showed that Beauveria sp. IPBCC.19.1499 and A. sclerotiorum IPBCC.19.1500 cultured at 50 mL PDB in Erlenmeyer 250 mL for 7, 14 and 21 days incubation by shaking at room temperature did not produce quinidine. This could be an excessive amount of oxygen that could inhibit quinidine production. Cultivation of fungi in liquid medium in static conditions was somewhat characterized by lack of oxygen and further there is a possibility of
stress in the form of the limited surface area of the medium. These conditions for by *Beauveria* sp. IPBCC.19.1499 and *A. sclerotiorum* IPBCC.19.1500 may induce the production of quinidine.

![Figure 2. Quinidine chromatogram on samples. (a-c) *Beauveria* sp.IPBCC.19.1499 at 7, 14 and 21 days of incubation as well as (d-f) *A. sclerotiorum* IPBCC.19.1500 at 7, 14 and 21 days of incubation. Remarks: the quinidine peak is shown by an arrow. The ordinate is absorbance (mAU) and axis is retention time (minutes).](image-url)
Table 1. Quinidine production by *Beauveria* sp. IPBCC.19.1499 and *A. sclerotiorum* IPBCC.19.1500 at different incubation periods.

| Sample          | Incubation period (days) | Quinidine concentration (mg/L) | Crude extract (mg/mL) | Dry biomass (mg) |
|-----------------|--------------------------|--------------------------------|-----------------------|------------------|
| IPBCC.19.1499   | 7                        | 2.873<sup>b</sup>              | 0.013<sup>a</sup>     | 263<sup>c</sup>  |
|                 | 14                       | 3.303<sup>b</sup>              | 0.017<sup>a</sup>     | 360<sup>a</sup>  |
|                 | 21                       | 4.125<sup>a</sup>              | 0.010<sup>a</sup>     | 303<sup>b</sup>  |
| IPBCC.19.1500   | 7                        | 1.275<sup>d</sup>              | 0.017<sup>a</sup>     | 137<sup>c</sup>  |
|                 | 14                       | 1.946<sup>c</sup>              | 0.013<sup>a</sup>     | 193<sup>d</sup>  |
|                 | 21                       | 3.055<sup>b</sup>              | 0.013<sup>a</sup>     | 303<sup>b</sup>  |

Remarks: the numbers in the same column followed by different letters are significantly different at α = 0.05

Table 2. Pearson correlation test between product of quinidine, dry biomass and crude extract.

| Sample                        | The crude extract ($r_p$) | Dry biomass ($r_p$) |
|-------------------------------|---------------------------|---------------------|
| *Beauveria* sp. PBCC.19.1499 | -0.581                    | 0.242               |
| *A. sclerotiorum* PBCC.19.1500| -0.787                    | 0.999*              |

Table 3. Pearson correlation between dry biomass production and crude extract weight

| Sample                        | the correlation value of the dry biomass with the crude extract weight |
|-------------------------------|------------------------------------------------------------------------|
| *Beauveria* sp. IPBCC.19.1499 | 0.491                                                                  |
| *A. sclerotiorum* IPBCC.19.1500| -0.271                                                                 |

5. Conclusion

*Beauveria* sp. IPBCC.19.1499 and *Aspergillus sclerotiorum* IPBCC.19.1500 produce quinidine based on the appearance of peaks on chromatograms that had the same retention time with the standard quinidine curve (Rt 1.892 ± 0.0125). The quinidine production was influenced by the type of fungi and the length of incubation tested. The longer the incubation time, the higher the quinidine concentration detected. The fungi that is cultured in a static condition promotes quinidine production.

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