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SYRINGIC ACID AND PHENAZINE PRODUCED BY AN ENDOPHYTIC *Pseudomonas aeruginosa* STRAIN G-111-0317 AND THEIR ACTIVITIES AGAINST *Ganoderma boninense*

Asam Siringat dan Fenazin yang Diproduksi oleh Endofitik *Pseudomonas aeruginosa* dan Aktivitasnya terhadap *Ganoderma boninense*

Bedah Rupaedah*, Nurlaila, Anisa Lutfia
Research Center for Applied Microbiology, National Research and Innovation Agency,
Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor, West Java 16911, Indonesia
*Email: beda001@brin.go.id

ABSTRACT

Syringic acid and phenazine possess antibiotic and antifungal properties, and have demonstrated effectiveness in inhibiting the colonization of Ganoderma boninense *on oil palm plants. Some bacteria, including* Pseudomonas aeruginosa *strain G-111-0317, are capable of producing syringic acid and phenazine. In this study, the culture extract of this bacterial strain was obtained from healthy oil palm plants growing in* G. boninense*-infected areas at Oil Plant Plantation Pematang Siantar, North Sumatra. The strain was cultured on Nutrient Broth (NB) medium, and the resulting culture filtrate was extracted using ethyl acetate (EtOAc) and concentrated under vacuum. The putative compounds were identified by LC-MS, employing syringic acid and phenazine as reference standards. Antifungal activity against* G. boninense *in vitro was observed in the EtOAc extract obtained after 8 hours and 24 hours of fermentation, with the 8-hour extract demonstrating the highest activity. These compounds hold promising potential as active agents in inhibiting basal stem rot disease in oil palm plants.*

Keywords: *Antifungi,* Ganoderma boninense*, phenazine,* Pseudomonas aeruginosa*, syringic acid*

ABSTRAK

Asam siringat dan fenazin merupakan antibiotik dan antijamur. Senyawa-senyawa tersebut telah terbukti efektif dalam menghambat kolonisasi *Ganoderma boninense* pada tanaman kelapa sawit. Beberapa bakteri memiliki kemampuan untuk memproduksi asam siringat dan fenazin. Senyawa bioaktif tersebut dilacak dari ekstrak kultur *Pseudomonas aeruginosa* strain G-111-0317 yang diisolasi dari tanaman kelapa sawit sehat yang tumbuh di daerah yang terinfeksi oleh *G. boninense* di Perkebunan Kelapa Sawit Pematang Siantar, Sumatra Utara. Galur bakteri dikultur dalam media Nutrient Broth (NB) dan filtrat kultur diekstraksi menggunakan etil asetat (EtOAc) dan dipekatkan secara in vocuo. Senyawa bioaktif diidentifikasi menggunakan LC-MS dengan menggunakan asam siringat dan fenazin sebagai standar. Ekstrak EtOAc dari fermentasi 8 jam dan 24 jam menunjukkan aktivitas antijamur terhadap *G. boninense* secara in vitro. Aktivitas terbaik ditunjukkan oleh ekstrak dari hasil fermentasi 8 jam. Senyawa-senyawa tersebut diharapkan menjadi agen aktif yang efektif dalam menghambat penyakit busuk pangkal batang pada tanaman kelapa sawit.

Kata Kunci: Antifungi, *Ganoderma boninense*, fenazin, *Pseudomonas aeruginosa*, asam siringat

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INTRODUCTION

Infectious diseases have severe economic consequences for Southeast Asian countries, especially Indonesia and Malaysia, where oil palm plantations are affected. To combat these diseases, various strategies have been employed, focusing on biological control techniques that utilize biological control agents (BCAs) and their metabolites (Muniroh et al. 2019, Supramani et al. 2022). Bacteria have emerged as promising biocontrol agents that produce antifungal compounds targeting *Ganoderma boninense* (Irma et al. 2018). Bacteria have rapid growth rates and the ability to adapt to adverse conditions, making them ideal candidates for disease control.

Extensive research has been conducted on *Bacillus*, *Pseudomonas*, and *Stenotrophomonas* strains, demonstrating their ability to produce antifungal compounds that inhibit basal stem rot disease caused by *G. boninense* in laboratory experiments and field trials (Lim et al. 2019, Ramli et al. 2016, Rupaedah et al. 2018, Sinpakone and Aryantha 2019, Suryanto et al. 2012). Recent investigations have uncovered novel antifungal compounds such as syringic acid (Chong et al. 2012a, Surendran et al. 2021), phenazine (Parvin et al. 2016, Thacharodi et al. 2021), benzoic acid, and salicylic acid (Fernanda et al. 2021, Surendran et al. 2018). These compounds effectively suppress *G. boninense*, showing promise as valuable tools in disease management strategies.

Syringic acid (SA), a phenolic compound found in phytochemicals, has various therapeutic applications in human diseases and bioactivities (Srinivasulu et al. 2018). Chong et al. (2012a) have identified a correlation between the application of syringic acid and enhanced resistance of oil palm trees to *G. boninense* during infection. SA, along with caffeic acid and 4-hydroxybenzoic acids, plays a defensive role in protecting oil palm trees against *G. boninense* (Chong et al. 2012b, Jee and Chong 2014). Field-scale application of a 90-100 µg/L dose of syringic acid, combined with chitosan, effectively prevents *G. boninense* colonization in root tissue (Chong et al. 2012a).

Phenazine, like SA, is a bioactive pigment that shows promise in controlling *G. boninense*. These compounds are produced by diverse strains of *Pseudomonas*, identified as beneficial microorganisms (BCAs) with antifungal activity (Parvin et al. 2020). Phenazines exhibit toxicity towards certain bacterial species and alter cellular activity in vivo, acting as an inhibitory mechanism (Parvin et al. 2020). Phenazine and its derivatives can be mass-produced through axenic culture and traced using chromatographic analysis after extraction and purification (Parvin et al. 2016, Lee et al. 2018). Studies on *P. aeruginosa* B6 by Lim et al. (2019) showed that the ethyl acetate extract exhibited better growth inhibitory activity against *G. boninense* compared to hexane and acetone. Additionally, Lee et al. (2018) reported that phenazine produced by *P. aeruginosa* UPMP3 at a concentration of 1,000 ppm effectively inhibits the growth of *G. boninense.*

Currently, researchers are investigating and formulating syringic acid and phenazine compounds, either individually or in combination, as chemical cocktails for potential future production as *G. boninense* biofungicides (Sahebi et al. 2017, Zhu et al. 2018). The goal is to develop effective and environmentally friendly solutions that can be scaled up for commercial application to control the spread of *G. boninense* and prevent basal stem rot disease in oil palm plants.

When evaluating beneficial microorganisms (BCAs), it is essential to consider multiple functional characteristics, such as the production of diverse bioactive metabolites. However, the ability to produce specific metabolites is limited to certain species and strains, depending on their source of isolation (Alexander et al. 2021). While there have been numerous studies on phenazines produced by various *Pseudomonas* members, information regarding bacterial strains capable of producing syringic acid in laboratory settings is currently lacking.

This study aims to investigate the production of syringic acid and phenazine compounds by an indigenous strain, *P. aeruginosa* G-111-0317, isolated from healthy oil palm plants in areas affected by basal stem rot caused by *G. boninense*. Identifying and characterizing these two compounds in this specific strain will contribute to validating its potential for future field-scale applications.

MATERIALS AND METHODS

Location and time

This research was conducted at the Laboratory for Biotechnology, National Research and Innovation Agency (Agency for the Assessment and Application of Technology) on July 2017 to July 2022.

Stock cultures

P. aeruginosa G-111-0317 was previously isolated from healthy oil palm plant tissue in an area known for *G. boninense* related diseases at Oil Plant Plantation Pematang Siantar, North Sumatra. The pathogenic fungus *G. boninense* strain SSU008 used in this study was obtained from PPKS Marihat, Simalugun Regency, North Sumatra, Indonesia. Bacterial stock cultures were rejuvenated using Nutrient Agar (NA) medium, a standard and widely used medium for bacterial cultures.

Broth fermentation and metabolite extraction

The fresh inoculum of *P. aeruginosa* G-111-0317 was cultured on Nutrient Broth (NB) media. The bacterial culture was harvested during the logarithmic growth phase until the death phase, which occurred at 24 hours. The population density of *P. aeruginosa* G-111-0317 was determined using a standard plate count method at each observation period (Bivi et al. 2010).

To extract the bioactive compounds, bacterial suspensions were centrifuged at 10,000×g for 10 minutes, and the supernatant was collected. Ethyl acetate (EtOAc) solvent was added to the supernatant in a 1:1 (v/v) ratio (Bivi et al. 2010). The mixture was vigorously shaken in a separating funnel for 15 minutes, resulting in the formation of two distinct layers: the upper layer consisting of the organic solvent and the lower layer containing the culture media and cell debris. The upper layer, containing the desired compounds, was carefully decanted and subsequently concentrated using a rotary vacuum evaporator. Finally, the concentrated extract was dried at room temperature.

Antifungal test

An inhibition test was conducted to assess the effectiveness of *P. aeruginos a* G-111-0317 culture extract against *G. boninense*. The modified cylindrical plate method, as described by Irma et al. in 2018, was employed by creating holes on potato dextrose agar (PDA) media using a cork borer. The ethyl acetate (EtOAc) extract derived from the bacterial culture was introduced into the well, achieving a concentration of 10,000 mg L-1. Subsequently, a 1 × 1 cm agar plug containing *G. boninense* was placed adjacent to the well containing the extract on the PDA medium. Nystatin was used as a positive control at the same concentration as the extract, while a negative control was conducted without the addition of the extract. The inhibition rate was assessed following the methodology outlined by Bivi et al. in 2010.

Detection of syringic acid and phenazine using LC-MS

The bioactive compounds present in the bacterial extracts were analyzed using liquid chromatography-mass spectrometry (LC-MS). For this analysis, 20 mg of the ethyl acetate (EtOAc) bacterial extract was mixed with methanol, homogenized using a vortex, and then centrifuged at 13,500 rpm for 10 minutes. The resulting clear supernatant solution (5 µL) was injected into an LC-MS system equipped with an Acquity UPLC BEH C18 1.7 µm column (2.1 × 50 mm). The elution was carried out at a flow rate of 0.3 mL/min using two mobile phases: A1 (0.1% formic acid in water) and B1 (0.1% formic acid in acetonitrile/MeCN).

The gradient conditions were set as follows: from 0 to 1 minute, 95% A1; from 6 to 7 minutes, 0% A1; from 7.5 to 9 minutes, 95% A1; from 0 to 1 minute, 5% B1; from 6 to 7 minutes, 100% B1; and from 7.5 to 9 minutes, 5% B1. All detectable peaks were analyzed using a Q-TOP MS system in the positive ion mode of electrospray ionization mass spectrometry (ESI-MS). The LC-MS profile was further examined and processed using Agilent ChemStation 4.3 software.

RESULTS AND DISCUSSION

To determine the maximum production or secretion of phenazine in the fermentation medium, the population density (CFU/mL) of

P. aeruginosa G-111-0317 was monitored over a 24-hour period (Figure 1). The logarithmic growth phase occurred between 4 and 8 hours, followed by a gradual increase from 12 to 16 hours. At the 16-hour mark, the growth peak was reached, with a population density of log 8.53 CFU/mL. Subsequently, the population density slowly declined towards the 24-hour mark.

Several factors can influence the growth rate of a bacterial strain, including strain-spec ific or genetic traits, the type of growth medium, and fermentation conditions, as highlighted by Ehrenberg et al. in 2013. Phenazine detection in the broth medium during the growth phase of *Pseudomonas* spp. can be visually observed through a color change in the post-fermentation stage. Parvin et al. (2016) reported that a medium turning blue-green or green indicates the presence of phenazine, along with a mixture of other pigments such as pyocyanin and phenazine-l-carboxylic acid.

The antifungal activity of EtOAc extracts obtained f rom dif ferent harvest periods of *P. aeruginosa* G-111-0317 against *G. boninense* was evaluated using a modified cylindrical method (Figure 2). The negative control (without EtOAc extract) did not exhibit any inhibition of *G. boninense* growth, while a slight inhibition was observed with the use of nystatin as a positive control. Notably, the EtOAc extracts displayed antifungal activity after 8 hours and 24 hours, resulting in a radial inhibition percentage of *G. boninense* greater than 80%. To investigate the presence of phenazine and syringic acid, the two f ractions were further analyzed using LC-MS.

A previous study by Lim et al. (2019) conducted on *P. aeruginosa* B6 revealed that the ethyl acetate extract exhibited superior inhibitory effects on the growth of

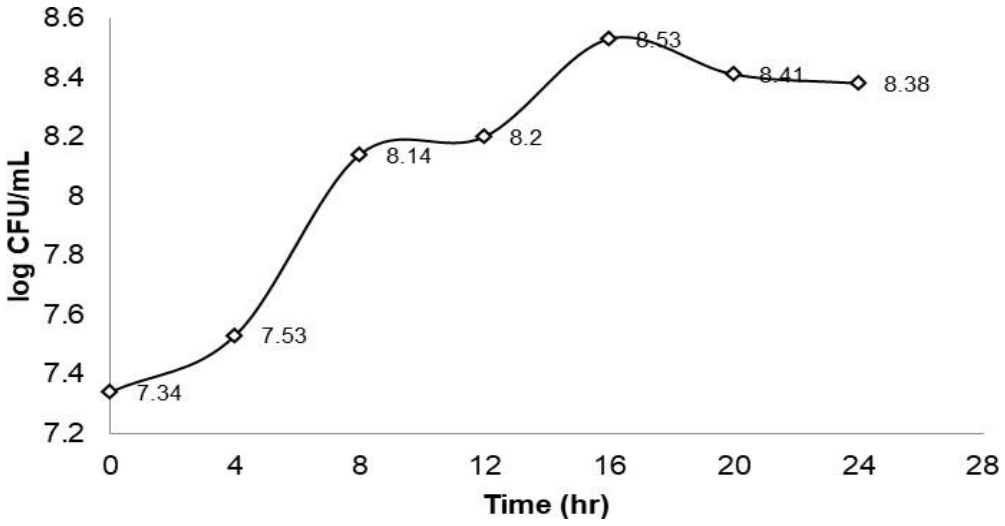

Figure 1. Growth profile of *P. aeruginosa* G-111-0317 on NB medium

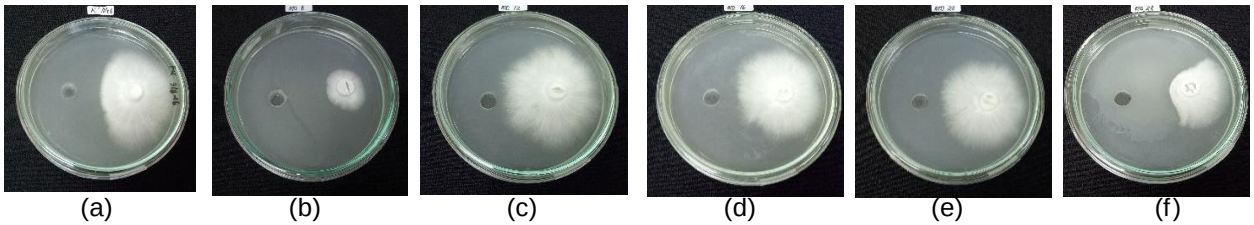

Figure 2. Antifungal activity of EtOAc extract of *P. aeruginosa* G-111-0317. (a) Nystatin; (b) 8 h; (c) 12 h; (d) 16 h; (e) 20 h; (f) 24 h.

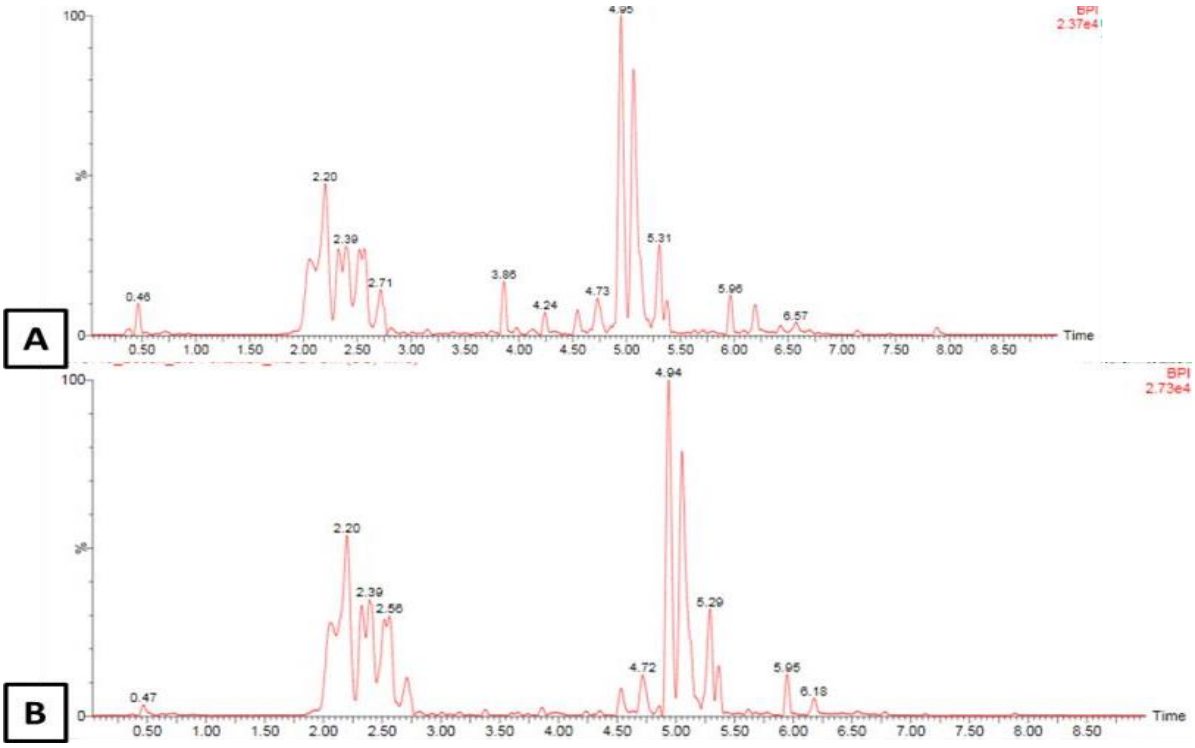

Figure 3. LC-MS spectrum of EtOAc extract of *P. aeruginosa* G-111-0317 harvested at (A) 8 h and (B) 24 h.

G. boninense compared to other solvents such as hexane and acetone. These findings corroborate our results, suggesting that the EtOAc extracts of *P. aeruginosa* G-111-0317 potentially contain phenazine or other antifungal metabolites.

The LC-MS analysis revealed the presence of various chemical compounds in the EtOAc extracts of *P. aeruginosa* G-111-0317, as indicated by the peaks in Figure 3. The molecular weights of syringic acid and phenazine were determined to be 198.17 and 180.21, respectively. Mass fragmentation analysis suggested that the 8 h extract possibly contained syringic acid, which appeared at a retention time of 0.20 min with an [M+H]+ m/z value of 199.1633. Phenazine was detected at a peak of 0.22 min with an [M+H]+ m/z value of 181.1340 (Figure 4). The retention time values for syringic acid and phenazine in the 24 h extract were also similar to those observed in the 8 h extracts (Figure 4).

A similar finding was reported by Hu et al. (2005) in their study on *P. aeruginos a* strain M-18, where LC-MS analysis indicated the presence of a phenazine moiety in the base peak at m/z 180. They validated this finding using UV-spectrum analysis. However, no comparable studies regarding the spectrum of syringic acid were found through LC-MS analysis. Our results consistently detected phenazine and syringic acid using LC-MS across different sampling periods. This method demonstrates high specificity and can accurately determine the molecular weight of target compounds.

Lim et al. (2019) also employed LC-MS to identify the major f raction of the EtOAc extract f rom *P. aeruginosa* B6, which was subsequently identified as 3-demethylubiquinone-9, a quinone antibiotic with limited information on its activity against phytopathogenic fungi, including *G. boninense*. To our knowledge, this is the f irst report on the detection of syringic acid produced by an endophytic bacterial strain, *P. aeruginosa* G-111-0317, isolated f rom oil palm. The f indings suggest that this strain could serve as a potential producer of multiple antifungal compounds. Further identification of other potential phenolic compounds and their derivatives would contribute to a better understanding of the specific strain or bacteria as a prominent biological control agent against *G. boninense* (Singh et al. 2017).

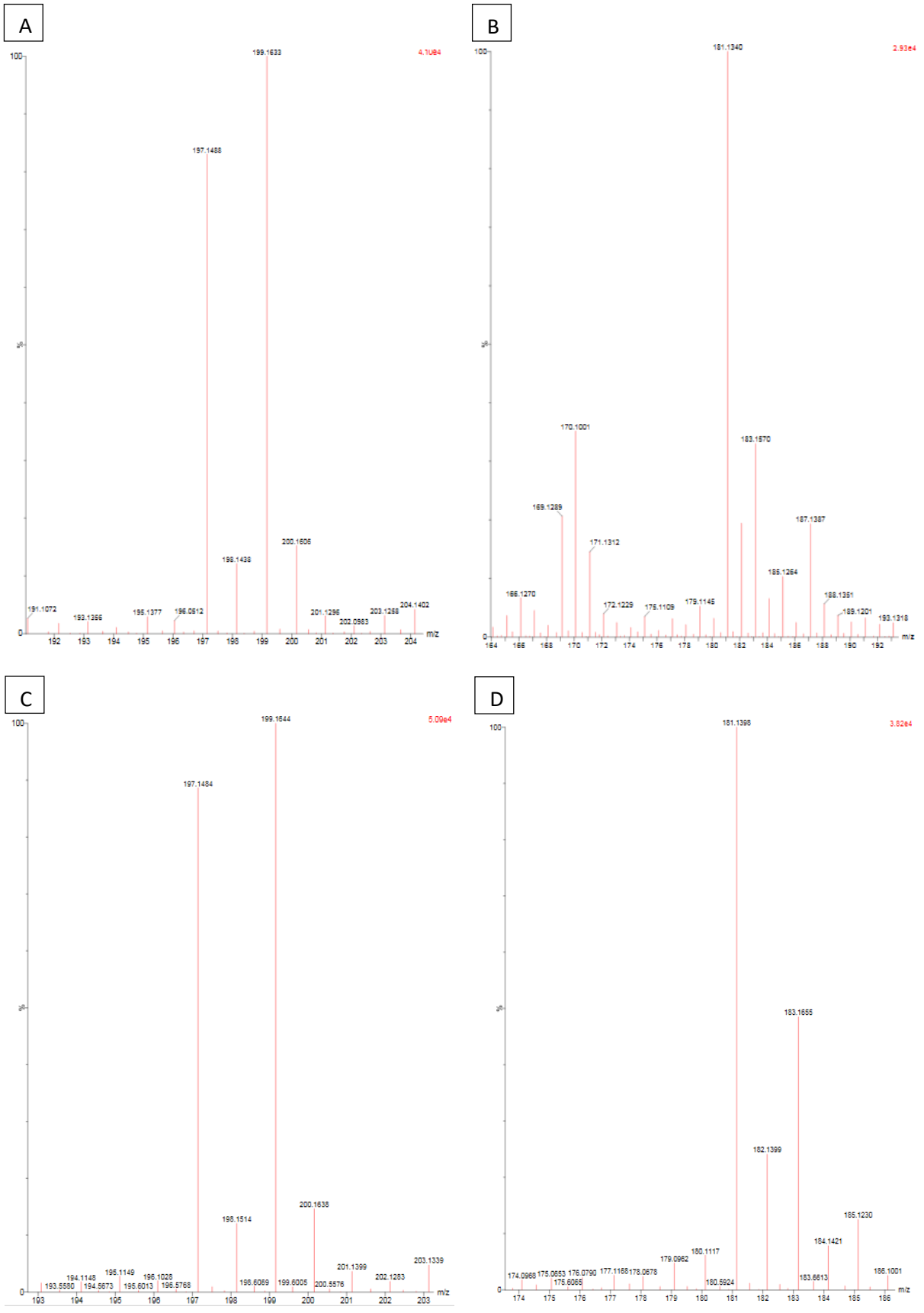

Figure 4. Mass fragmentation of EtOAc extract of *P. aeruginosa* G-111-0317 at 8 h (A) syringic acid (B) phenazine and at 24 h (C) syringic acid and (D) phenazine

CONCLUSION

The EtOAc extract of *P. aeruginosa* G-111-0317, harvested at 8 h and 24 h of incubation, showed the presence of phenazine and syringic acid. These results suggest that these specific sampling periods could be targeted for large-scale production of these compounds using fermentation techniques. Additionally, the identified compounds exhibited potential inhibitory effects against *G. boninense*, highlighting their potential as antifungal agents.

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