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Identification of Antimycobacterial from Actinobacteria (INACC A758) Secondary Metabolites using Metabolomics Data

(Pengenalpastian Antimikobakteria daripada Metabolit Sekunder Aktinobakteria (INACC A758) menggunakan Data Metabolomik)

MAYA DIAN RAKHMAWATIE[1,2], MUSTOFA[3], PUSPITA LISDIYANTI[4], WORO RUKMI PRATIWI[3] & TRI WIBAWA[5,*]

[1]*Doctoral Program of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[2]*Department of Biomedical Sciences, Faculty of Medicine, Universitas Muhammadiyah Semarang, Semarang 50273, Indonesia*
[3]*Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*
[4]*Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong, Bogor 16911, Indonesia*
[5]*Department of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

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ABSTRACT

Actinobacteria produce active secondary metabolite with medicinal properties, such as antibacterial or anticancer. However, there are some reports about the difficulties in discovering novel secondary metabolites. Therefore, the need for a new approach is obvious. Several factors such as types of nutrients in the culture media or different solvents used for extraction have been proven to influence the Actinobacteria secondary metabolite production. In this study, a combination of culture media optimization and metabolites fingerprint analysis were applied to identify antimycobacterial active compounds from Actinobacteria (InaCC A758). Five culture media were used in the secondary metabolite production of the Actinobacteria. The metabolite fingerprinting was carried out by analyzing the secondary metabolite profile extracted from culture media optimization using UPLC-MS. Multivariate analysis, i.e. cluster analysis and principal component analysis (PCA) was applied. The result showed that a unique antimycobacterial compound candidate against *Mycobacterium smegmatis* was produced by SYP media cultured InaCC A758 (MIC 6.25 µg/mL).

Keywords: Actinobacteria; antimycobacterial; culture optimization; metabolite fingerprint; secondary metabolites

ABSTRAK

Aktinobakteria menghasilkan metabolit sekunder aktif dengan sifat perubatan, seperti antibakteria atau antikanser. Walau bagaimanapun, terdapat beberapa laporan tentang kesukaran untuk menemui metabolit sekunder yang novel. Oleh itu, keperluan untuk pendekatan baru adalah jelas. Beberapa faktor seperti jenis nutrien dalam media kultur atau pelarut berbeza yang digunakan untuk pengekstrakan telah terbukti mempengaruhi pengeluaran metabolit sekunder Aktinobakteria. Dalam kajian ini, gabungan pengoptimuman media kultur dan analisis cap jari metabolit digunakan untuk mengenal pasti sebatian aktif antimikobakteria daripada Aktinobakteria (InaCC A758). Lima media kultur digunakan dalam penghasilan metabolit sekunder Aktinobakteria. Cap jari metabolit telah dijalankan dengan menganalisis profil metabolit sekunder yang diekstrak daripada pengoptimuman media kultur menggunakan UPLC-MS. Analisis multivariat, iaitu analisis kelompok dan analisis komponen utama (PCA) telah digunakan. Keputusan menunjukkan bahawa sebatian antimikrobakteria unik terhadap *Mycobacterium smegmatis* dihasilkan oleh media SYP yang dikulturkan dengan InaCC A758 (MIC 6.25 µg/mL).

Kata kunci: Aktinobakteria; antimikobakteria; cap jari metabolit; metabolit sekunder; pengoptimuman budaya

INTRODUCTION

Actinobacteria is a phylum that occupies the largest taxonomy in the bacterial domain (Singh & Dubey 2018). Actinobacteria are Gram-positive bacteria with high G + C DNA, and have been proved to produce biologically active secondary metabolites with medicinal properties,

such as antibacterial or anticancer (Barka et al. 2016; Bérdy 2012; Lahlou 2013). Unfortunately, there was a decline in the number of novel secondary metabolite findings from Actinobacteria (Gaudêncio & Pereira 2015; Mammo & Endale 2015). Therefore, a new approach using optimization of culture parameters is needed to find a new secondary metabolites from Actinobacteria (Bode et al. 2002; Romano et al. 2018; Zhu et al. 2014;). Apart from the culture process, extraction can also affect the process of obtaining secondary metabolites with different levels of polarity (Sharma et al. 2011). During the extraction process, it should also be noted that secondary metabolites can be found not only in microbial cells (intracellular) but also can be released into the culture media (extracellular). Secondary metabolites produced in the optimization of culture and extraction process can differ in terms of biological activity, or the different classes of compounds (Rajan & Kannabiran 2014; Retnowati et al. 2018).

Meanwhile, extracts produced from a culture of Actinobacteria are difficult to evaluate due to the diversity of its compounds. Methods of analyzing secondary metabolite profiles of Actinobacteria can be performed using tandem liquid chromatography (LC) separation techniques with mass detection using spectroscopy (MS). The LC-MS analysis method can be used to find the novel microbial secondary metabolites by matching LC-MS profile data with a natural product database (Hamedi et al. 2015; Zaher et al. 2015). If a natural product database is not available, multivariate statistical analyses such as Principal Component Analysis (PCA) or cluster analysis of LC-MS metabolite profile data can be used to predict novel secondary metabolites (Cordella 2012).

This research optimized the selection of culture media and solvents used for extraction in order to stimulate the unique and bioactive secondary metabolite production of Actinobacteria (InaCC A758). Antimycobacterial activity analysis of the extracts was conducted and combined with the results from the metabolite fingerprint analysis method, in order to determine the extract candidates with unique profiles to be explored further. The antimycobacterial activity was carried out by testing the secondary metabolite extract of InaCC A758 against *Mycobacterium smegmatis*, the Rapidly Growing Mycobacteria (RGM). *Mycobacterium smegmatis* usually nonpathogenic, but an increase in the number of people with immunodeficiency can increase the risk of infection. Rapidly growing mycobacteria are also difficult to eradicate because of their higher natural resistance to antimicrobial (Brown-Elliott et al. 2012; van Ingen et al. 2012).

Materials and Methods

Research subjects

The Actinobacteria used in the study was InaCC A758 isolate which is a collection of InaCC (Indonesian Culture Collection) at Indonesian Institute of Science, Cibinong, Indonesia. InaCC A758 was isolated from rhizosphere soil in Pramuka Island, Jakarta, Indonesia. Furthermore, the isolate was identified as *Streptomyces badius* (Setiawati et al. 2021). Antimycobacterial activity test of the InaCC A758 extract from the culture and extraction optimization was carried out on *M. smegmatis* MC2 155 (ATCC® 700084™).

Culture media

The culture media used to optimize InaCC A758 secondary metabolites production were SYP broth (1% soluble starch, 0.4% yeast extract, and 0.2% bacto peptone), ISP-2 (1% malt extract, 4% yeast extract, and 4% glucose anhydrate in aquadest), ISP-4 (1% soluble starch, 0.2% $(NH_4)_2SO_4$, 1% K_2HPO_4, 1% $MgSO_4.7H_2O$, 2% NaCl, and 20% $CaCO_3$ in aquadest) (Kiranmayi et al. 2011), SGG (10% soluble starch, 10% glucose, 10% glycerol, 2.5% corn steep powder, 5% bacto peptone, 2% yeast extract, 1% NaCl, and 30% $CaCO_3$ in aquadest), and ASW-A (20% soluble starch, 10% glucose, 5% bacto peptone, 5% yeast extract, and 5% $CaCO_3$ in artificial sea water). ASW (Artificial Sea Water) composition per 1 L aquadest are 24.6 g NaCl, 0.67 g KCl, 1.36 g $CaCl_2.2H_2O$, 6.29 g $MgSO_4.7H_2O$, 4.66 g $MgCl_2.6H_2O$, and 0.18 g $NaHCO_3$ (Adnani et al. 2015).

Culture process of InaCC A758

Seven days old InaCC A758 from SYP agar were transferred to be pre-cultured first in SYP broth for 48 h. After completion of the pre-culture, the InaCC A758 then transferred to the culture process using baffled flasks (Duran®) containing each production media. The volume of pre-culture added to the culture process was 10% of the total amount of culture media, and the total amount of culture media was 20% of the maximum volume of the baffled flask used (Narayana & Vijayalakshmi 2008; Wang et al. 2010). The temperature for the pre-culture and culture process were 30 °C, respectively, while the shaker orbital (Biobase Ref. BJPX-100B) agitation speed was 135 rpm (de Oliveira et al. 2010). After 3

days of the culture process, the bacterial suspension was transferred to a centrifuge tube, then centrifuged at a speed of 6000 g for 15 min (Sorvall™ Primo™ R). The supernatant was separated from the cells and followed by an extracellular extraction process, while bacterial cell deposits were prepared for intracellular extraction (Sengupta et al. 2015).

OPTIMIZATION OF METHODS AND SOLVENT VARIATIONS FOR THE EXTRACTION

For intracellular extraction, precipitated InaCC A758 cell was added with methanol up to twice the volume of the precipitate, then shaken several times. After that, the cell suspension in methanol (Merck Ref. 106009) was re-centrifuged at 6000 g for 15 min. The methanol supernatant from the centrifugation results was taken, then was extracted by liquid-liquid extraction using 1:1 v/v chloroform (Merck Ref. 102445) to obtain methanol-chloroform extract (Hoshino et al. 2014).

For extracellular extraction, the supernatant obtained from centrifugation of culture process was then divided into two parts, and each part was extracted using liquid-liquid extraction method with the addition of ethyl acetate (Merck Ref. 109623) or chloroform 1:1 v/v. Organic solvents in all extracts were dried with a rotary evaporator (Ika® RV 10) at 40 °C (Sengupta et al. 2015). The dried extract was dissolved with methanol and stored at 2-8 °C for metabolite fingerprint analysis and antimycobacterial activity test.

ANTIMYCOBACTERIAL ACTIVITY TEST OF EXTRACTS

Mycobacterium smegmatis MC2 155 was prepared into bacterial suspension using Mueller Hinton (MH) Broth (Himedia Ref. M391), and adjusted with sterile distilled water to a 1.0 McFarland standard. After entering the test plate, the final concentration of *M. smegmatis* inoculum was 5×10^5 CFU/mL, according to the guideline for susceptibility testing of nontubercolous mycobacteria from document M24-A2 (Woods et al. 2011).

For antimycobacterial activity testing, the extract from media culture and extraction optimization were prepared using 5% DMSO (Sigma Aldrich Ref. M81802). The final test concentrations for the extracts used were obtained by serial two-fold dilutions ranging from 6.25 to 100.0 µg/mL. Resazurin Microplate Assay (REMA) methods were used for the antimycobacterial activity testing, and stained with resazurin (Sigma Aldrich Ref. 7017) after 2-3 days incubation, and were re-incubated for an additional 1 h at 37 °C (Rakhmawatie et al. 2019).

ULTRA-PRESSURE LIQUID CHROMATOGRAPHY-MS ANALYSIS

Actinobacteria extracts from culture media and extraction optimization were tested for metabolites profiles using UPLC-MS. Each extract was dissolved using methanol to a concentration of 1,000 µg/mL (Djinni et al. 2013). The sample was filtered using a 0.22 µm nylon membrane filter syringe to prevent clogging of the column and improve the separation process on chromatography (Ito et al. 2011).

The metabolite profile of the InaCC A758 extract was analyzed using a reversed-phase chromatography system of the Ultra Pressure Liquid Chromatography (UPLC, Waters™ Acquity™ UPLC™ H-Class) instrument with C_{18} column 1.7 µm; 2.1 × 50 mm (Acquity UPLC®, BEH). The volume of the extract injected into the column was 0.2 µL. The mobile phase used: A (0.1% formic acid in H2O) and B (0.1% formic acid in acetonitrile). The extract was eluted using 10% mobile phase B, followed by a linear gradient from 10 to 100% mobile phase B within 20 min, with a mobile phase rate of 0.4 mL/min. MS analysis was performed by positive mode electrospray ionization, with a capillary voltage of 3.5 kV and a cone voltage of 30 V, a temperature of 500 °C. The MS instrument used was quadrupole mass analyzer (Waters™ Xevo™ TQD), with a range of m/z scan from 50 to 1350 (Schrey et al. 2012).

STATISTICAL ANALYSIS

In this study, UPLC-MS data were used to verify the peak chromatogram for any changes in secondary metabolites, then the peaks were deconvoluted to provide discrete features corresponding to specific retention times and m/z pairs (Derewacz et al. 2013). Determination of retention time and m/z values were done manually with a tolerance value 0.05 units for m/z 0.2 min for retention times (Perrot-Dockès et al. 2018).

Multivariate statistical analysis was performed using Minitab 16.0. Cluster grouping of secondary metabolites of InaCC A758 extract was determined based on similarity test values with the Hierarchical Cluster Analysis. Finally, principal component analysis was used to examine the secondary metabolites responsible for the difference between extracts, using the covariance matrix method.

Results and Discussion

Culture media used for InaCC A758 fermentation and also different solvents used for extraction affect the color of the extract produced. The optimization process can also cause differences in biological activity and chromatogram patterns of the extracts produced (Table 1). Then, the analysis continued by grouping secondary metabolite profiles using cluster analysis.

In this study, the chromatogram of secondary metabolite profile of InaCC A758 extract was constructed using a combination of tandem liquid chromatography with ESI-MS. UPLC used in this study has advantages in terms of resolution compared to standard LC, since the separation of compounds is the basic of the success of metabolite profiling (Wolfender et al. 2019). In addition, ion suppression problems related to metabolite co-elution are less common with UPLC (Guillarme et al. 2010). In general, reversed-phase liquid chromatography is used for metabolite profiling, due to its compatibility with ESI-MS or APCI detection methods.

Cluster analysis is used to group secondary metabolite profiles based on similarities in peak LC-MS chromatogram patterns (Cumsille et al. 2017). While PCA is another multivariate analysis that can be used efficiently to identify buckets in data points that are responsible for the discrimination of samples within the entire dataset. In this study, PCA was used more as an exploratory tool than a prediction model (Forner et al. 2013).

From the cluster analysis result and score plot of PCA, secondary metabolite profiles from ISP-2 and ISP-4 extracts group were separated from SYP and ASW-A extracts group, meanwhile the SGG extract was outside both groups (Figure 1). It can be concluded that the variety of culture media used, lead to the differences in the profile of secondary metabolite produced by InaCC A758. This is in line with several previous reports (Liu et al. 2010; Tormo et al. 2003).

To determine the secondary metabolites that cause differences in the secondary metabolite profile of InaCC A758 extract, an outlier plot of PCA was performed (Figure 2). The most unique compound produced due to culture media optimization is a compound with m/z value of 940.30, produced by ISP-4 cultured InaCC A758. ISP-4 is considered as the most different media due to its phosphate content. Phosphate minerals are reported to increase bioactive metabolite production in *Streptomyces cheonanensis* VUK-A and other Actinobacteria (Mangamuri et al. 2012). However, the unique compound produced using ISP-4 was unable to increase the extract's biological activity against *M. smegmatis*.

Remarkably, the extract produced by the InaCC A758 isolate had the highest activity when cultured using SYP media (Table 1). The multivariate PCA analysis showed that the extract of InaCC A758 cultured in SYP media had the potential to produce unique compounds with m/z values of 1255.56. Overall, it can be concluded that the SYP media is the most optimal media for InaCC A758 to produce unique *M. smegmatis* growth inhibitory compound.

In addition to phosphate, carbon and nitrogen are also proven to affect the secondary metabolite production of Actinobacteria. Nitrogen in peptone or yeast extract, are reported to increase the production of antibiotic by *Streptomyces* sp. (Al-Ghazali & Omran 2017). Secondary metabolite with antimycobacterial activity is produced by InaCC A758 cultured using nitrogen sources from peptone and yeast extract (SYP, SGG, and ASW-A culture media). It seems that nitrogen as a source of amino acids affect the products synthesized using the NRPS pathway (Romano et al. 2018).

Complex carbons such as starch or dextrin are more preferable than glucose or glycerol in increasing the production of Actinobacteria secondary metabolite. Glucose is rapidly used by microbial cells, but increases cell growth more than secondary metabolite production (Al-Ansari et al. 2020; Jacob et al. 2017; Romero-Rodríguez et al. 2018; Ruiz et al. 2010). In this research, only ISP-2 did not use complex carbon sources, and the resulting extract did not exhibit antimycobacterial activity or unique potential secondary metabolites. Nonetheless, other research reported that *Streptomyces* sp. cultured using ISP-2 positively produce anti-candida components, and the anti-candida activity was reduced when carbon glucose was removed from ISP-2 culture media (Escher et al. 2016).

The optimization continued with the selection of organic solvents for the extraction of secondary metabolites. Extraction solvents have been shown to cause diversity in the profile of secondary metabolites produced by SYP media cultured InaCC A758 (Figure 3). Methanol and chloroform (devoted for intracellular extracts) have the biggest difference from ethyl acetate (EA) and chloroform (CHL) (devoted for extracellular extracts). An outlier plot of PCA was performed to determine the secondary metabolites that cause differences in the secondary metabolite profile of InaCC

A758 extract (Figure 4). From the result of SYP media cultured InaCC A758, solvents for the extraction of secondary metabolites did not significantly affect their antimycobacterial activity (Table 1). However, a unique *M. smegmatis* inhibitory compound with m/z value of 1255.56 which was produced by SYP media cultured InaCC A758, can only be extracted using ethyl acetate.

The metabolite fingerprinting method combined with biological activity test can be used efficiently to choose any extracts with the potential to have unique compounds with biological activity (Happyana et al. 2012; Wolfender et al. 2015). For example, predictions of similar compounds related to their antimicrobial activity were reported by using metabolite profiling analysis combined with the results of antimicrobial activity tests from extracts produced by *Aspergillus oryzae* (Son et al. 2018). In this study, a combination of biological activity test and metabolite profiling analysis can be applied to predict unique secondary metabolites of Actinobacteria in the antimycobacterial screening process.

TABLE 1. Antimycobacterial activity of InaCC A758 extracts produced in various culture media and extraction solvent optimization

Culture media and extraction solvent optimization	MIC (µg/mL)		
	Ethyl acetate	Chloroform	Metanol-chloroform
SYP	6.25	6.25	3.125
ISP-2	> 100	> 100	> 100
ISP-4	> 100	50	> 100
SGG	6.25	100	25
ASW-A	6.25	50	0.78

Notes: The MIC values written are mode values from the results of the antimycobacterial test carried out in triplicates

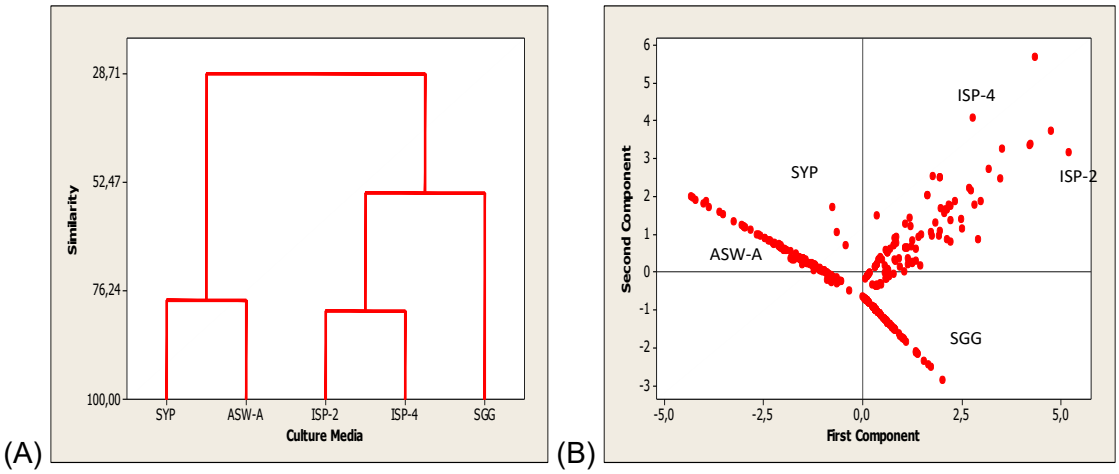

FIGURE 1. (A) Dendogram generated from cluster analysis of secondary metabolites extracted from InaCC A758 cultured in different media; (B) score plot from principal component analysis (PCA) of secondary metabolite profile of InaCC A758 extract produced in various culture media

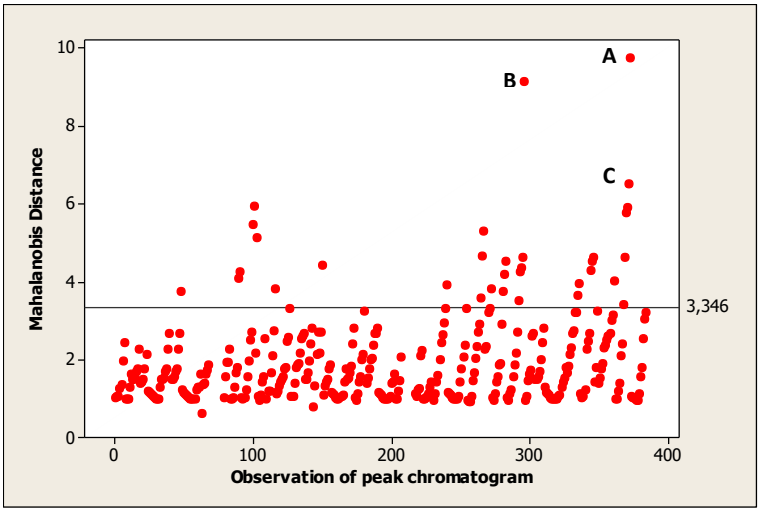

FIGURE 2. Outlier plot constructed from principal component analysis (PCA) secondary metabolite profile of InaCC A758 extract in various culture media

Note: (A) compounds with m/z 940.30 and RT 16,081 min resulted from ISP-4 culture media; (B) m/z compounds 1255.56 and RT 11,867 min resulted from SYP culture media; and (C) m/z compounds 619.53 and RT 16,081 min produced from ISP-4 culture media

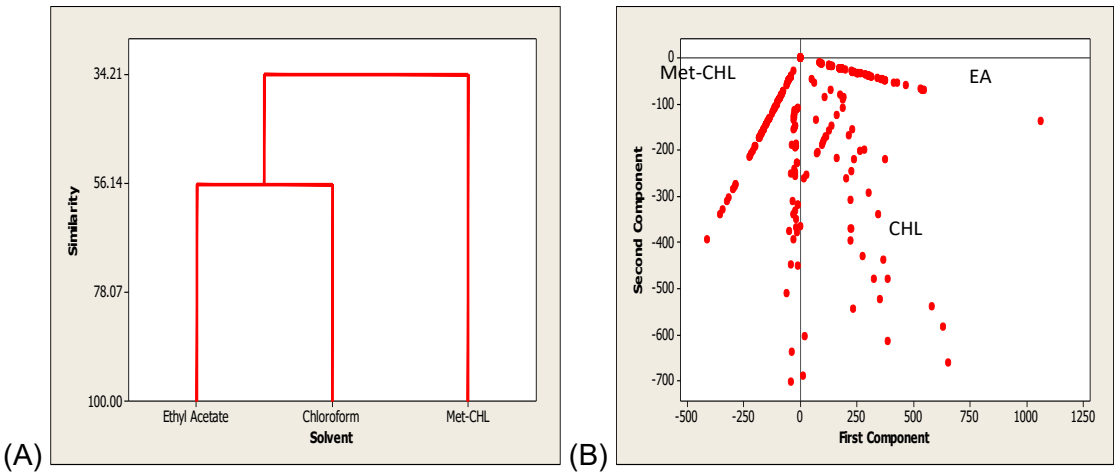

FIGURE 3. A) Dendogram generated from cluster analysis of secondary metabolite profile of SYP media cultured InaCC A758 extract using various organic solvent; (B) score plot from principal component analysis (PCA) of secondary metabolite profile of SYP media cultured InaCC A758 extract using various organic solvent

Note: EA (ethyl acetate); CHL (Chloroform); and Met-CHL (Methanol-Chloroform)

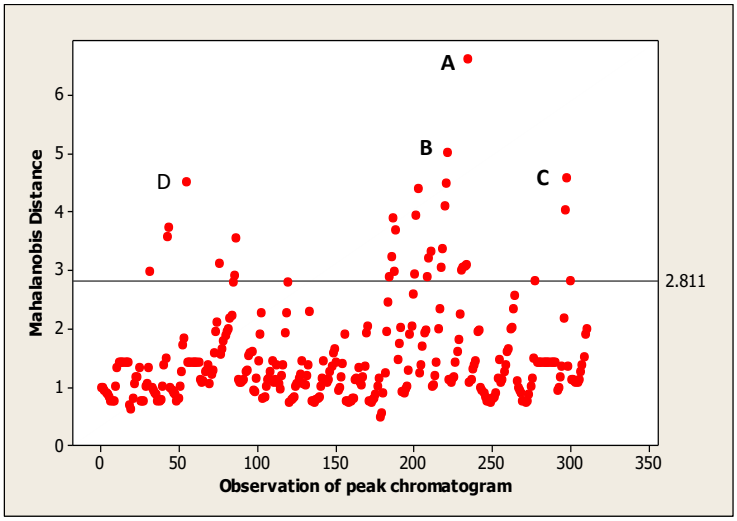

FIGURE 4. Outlier plot constructed from principal component analysis (PCA) secondary metabolite profile of SYP media cultured InaCC A758 extract in a various organic solvents

Note: (A) compounds with m/z 1255.56 and RT 11.867 min which are produced from extraction using ethyl acetate, (B) compound m/z 632.49; 683.36 and RT 11.227 min which are produced sequentially from extraction using ethyl acetate; chloroform, (C) m/z compound 678.49 and RT 16.081 min produced from extraction using chloroform, and (D) m/z compound 814.59 and RT 3.607 min produced from extraction using methanol-chloroform

CONCLUSION

A combination of culture media optimization and metabolites fingerprint analysis can be used to select candidate compound that has antimycobacterial activity against *M. smegmatis*. Based on the result of multivariate analysis, unique compound (m/z values of 1255.56) with the highest antimycobacterial activity (MIC 6.25 µg/mL) produced by SYP cultured InaCC A758 and extracted using ethyl acetate.

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*Corresponding author; email: twibawa@ugm.ac.id