

Validation of TLC densitometry method for the quantitative determination of alkaloid in fermented endophytic fungi extract *Phyllanthus niruri* Linn

Rollando Rollando^{*1}, Sandra Dewi Tansil¹, Eva Monica¹,
Yuyun Yuniati², Leny Yuliati²

¹Program of Pharmacy, Faculty of Science and Technology, Ma Chung University, Malang
Jl. Villa Puncak Tidar N-01, Malang 6515

²Program of Chemistry, Faculty of Science and Technology, Ma Chung University, Malang
Jl. Villa Puncak Tidar N-01, Malang 65151

Submitted: 12-02-2109

Reviewed: 27-03-2019

Accepted: 27-05-2019

ABSTRACT

Endophytic fungi can produce compound that similar with the host, so it can be used as mass production of compound. The optimal and valid assay method is needed to obtain the proper culture condition, which one using densitometric Thin Layer Chromatography (TLC). This research used 11 samples of endophytic fungi culture extract from *Phyllanthus niruri* within various culture condition. Optimization of mobile phase was done using 3 kinds of mobile phase mixture, chloroform: ethyl acetate: methanol (8:8:4); chloroform: ethyl acetate: methanol: NH₄OH (8:8:4:0.005); chloroform:ethyl acetate:NH₄OH (8:8:0.005). Validation method measured using some parameters such as linearity, accuracy, and precision. The result of optimization and validation showed that TLC densitometry method can be used for measuring alkaloid level within the endophytic fungi extract of *P. niruri* using chloroform: ethyl acetate: NH₄OH (8:8:0.005), with R value is 0.977, percentage of recovery is between 90–110, and RSD ≤7% on each concentration series. While the proper condition of endophytic fungi culture is using sucrose as carbon source and at pH 6 condition. The content of alkaoid reached 25.9 ± 1.4 mg 100 g⁻¹ fresh weight.

Keywords: validation, *Phyllanthus niruri*, TLC densitometry, alkaloid

***Corresponding author:**

Rollando Rollando

Program of Pharmacy, Faculty of Science and Technology, Ma Chung University, Malang
Jl. Villa Puncak Tidar N-01, Malang 6515

Email: ro.llando@machung.ac.id

INTRODUCTION

Endophytic fungi are fungi that colonize healthy plant tissue without causing symptoms or disease (Souza *et al.*, 2011). This fungi can produce compounds that are similar, even identical to the compounds produced by the host (Selim *et al.*, 2012). Almost all plants have endophytic fungi (Rodriguez *et al.*, 2009). One example is the meniran plant (*Phyllanthus niruri*) with its endophytic fungi from the genus *Fusarium*. Meniran plants contain various compounds, some of which are lignans, glycosides, alkaloids, elagitanin, terpenes, and phenylpropanoids (Colpo *et al.*, 2014).

The compounds contained in meniran plants have various pharmacological effects. Based on research conducted in mice, extracts from meniran plants can reduce blood sugar levels while protecting the pancreas with its antioxidant effects (Okoli *et al.*, 2010). Other studies carried out using endophytic fungi *Fusarium sp.* from meniran plants showed antibacterial activity than diethyl ether fractions, antioxidants in 96% ethanol fraction, and cytotoxic effects on diethyl ether fractions (Rollando *et al.*, 2017). The various pharmacological effects produced show the potential of meniran plants as anticancer, antibacterial and antidiabetic drugs (Rollando and Prilianti, 2018).

The nature of endophytic fungi that are capable of producing compounds similar to their hosts provides an opportunity to produce massively active compounds. However, from the results of the study the results of extracts obtained from endophytic fungi were still very small. This is evident from the results of a study conducted by extracting 100 g of endophytic fungal mycelium *Fusarium oxysporum* from the leaves of *Dysoxylum binectariferum* with only 295.83 µg of extract (Kumara *et al.*, 2014). The results of this small extract make it important to find a way to get the extract from endophytic fungi culture to the maximum (Rollando, R, 2018).

The results of extracts from endophytic fungi can be obtained maximally if cultured using effective methods while maintaining their ability (Murphy *et al.*, 2015). Determination of the results of a optimum culture for the acquisition of the maximum compound can not be separated from the measurement of levels in the extract obtained. Determination of compound levels requires the right method to determine the level of the compound to be measured.

Thin layer chromatography densitometry is one method that can be used for quantitative analysis (Yuniati and Rollando, 2018). Thin layer chromatography densitometry is a simple method that is suitable for analysis with large sample variations. In addition, the costs incurred by thin layer chromatography densitometry are less than those of HPLC (Yuniati *et al.*, 2018). Based on research on qualitative and quantitative analysis of protopin on *Fumaria sp.* using the HPLC method and thin layer chromatography densitometry showed that thin layer chromatography densitometry was faster, cheaper, and easier to determine protopin levels than HPLC (Vrancheva *et al.*, 2012).

The accuracy of a method in measuring levels can be determined by optimization and validation of the method used. Until now there is still no valid method for measuring alkaloid levels in endophytic fungi extracts of *Fusarium sp.* from *P. niruri* Linn leaves. Therefore, research is needed on the optimization and validation of alkaloid content determination methods in *Fusarium sp.* Fungi extract culture from *P. niruri* Linn leaves. to get the right leveling method.

MATERIALS AND METHOD

Materials

The ingredients were endophyte fungi from *Fusarium sp.*, fungal growth media PDA (Potato Dextrose Agar), PDB (Potato Dextrose Broth), NaCl, NaNO₃, HCl, NaOH, Glucose, Lactose, Dextrose, and Fungerin.

Mobile phase optimization

The mobile phase made with a mixture of chloroform, ethyl acetate, methanol and ammonium hydroxide with various comparisons such as in Table I. The mobile phase is inserted in the chamber. Before being used for development, the mobile phase is saturated by shaking it in the chamber.

Table I. Composition of mobile phase

| Mobile Phase | chloroform | ethyl acetate | methanol | NH ₄ OH |
|--------------|------------|---------------|----------|--------------------|
| A | 8 | 8 | 4 | - |
| B | 8 | 8 | 4 | 0,005 |
| C | 5 | 8 | - | 0,005 |

Standard Stock solution

Stock solutions are made with fungerin (isolated with preparative HPLC) with a concentration of 2000 ppm. The solution is made by weighing 4 mg of fungerin, then dissolved with methanol in a 2 mL microtube.

Standard solution series

The standard solution series is made in six types of concentration, 2000 ppm; 1800 ppm; 1600 ppm; 1400 ppm; 1200 ppm; 1000 ppm. The solution is made by dilution from the stock solution.

Determination of the maximum wavelength

Series standard solution spotted on the stationary phase and then elution with a mobile phase mixture. Development is done as high as 8 cm, then immediately dried and read absorbance at a wavelength of 200-800 nm wavelength range of 50 nm.

Sample preparation

Each extract of the sample obtained was dissolved with methanol with a concentration of 2000 ppm in a micro tube for the liquid to be bottled together with internal standard solutions.

Mobile phase optimization

The standard solution series that have been made are spotted in the stationary phase. Then developed in each mix of mobile phases. Development is done as high as 8 cm, and immediately dried to be measured at the maximum wavelength that has been obtained. The results of the development of samples from each mobile phase mixture compared to R_f and the chromatogram were standardized, then calculated the Asymmetry factor from the peak of the chromatogram obtained.

Validation method**Specificity**

Sample and standard solutions are analyzed by the optimal method, the R_f data obtained is then compared.

Linearity

Linearity studies are carried out using the six standard solution series. Each standard solution was bottled on the same stationary phase plate (each concentration was replicated three times), then developed with the optimal mobile phase. The development results are then read at the maximum wavelength. The results of the peak area are plotted with the alkaloid levels obtained so that the calibration curve and regression equation are obtained.

Accuracy

Accuracy test is calculated as percent recovery from each standard solution concentration that has been measured for the calibration curve.

Precision

The precision test is calculated by percent RSD from each standard solution concentration that has been measured for the calibration curve.

Measuring the levels of alkaloids in the sample

The optimal and valid level determination method is carried out on each sample of fungi culture (0.3% salinity, 0.2% salinity; 1% carbon source from glucose, maltose, starch, sucrose, fructose; nitrogen source from NaNO₃; and pH conditions 7, 6 and 5). After that the AUC results obtained are plotted in the equation of the calibration curve to calculate the alkaloid levels of each sample.

Data Analysis

Separation data from alkaloid in the extract would analyze with some parameters:

1) Choosing the most pointed peak of the chromatogram, then the results of the resolution of the peak are calculated using the equation described in the previous chapter, 2) Seeing the similarity of R_f from sample and standard, 3) Calculate the resolution of each mobile phase with the chromatogram peak of the alkaloid and other compounds beside it using the equation described in the previous chapter, 4) Calculate the correlation coefficient and percentage of recovery from concentration values obtained with 2 replications using the optimum mobile phase obtained, 5) Calculating the alkaloid levels in each sample using the calibration curve equation that has been obtained with Microsoft Excel.

RESULTS AND DISCUSSION

Mobile phase optimization

Optimization of separation in chromatography relates to the determination of the optimum conditions in separation. In Thin Layer Chromatography the most important thing in optimization is the selection of the mobile phase component. Determining the optimum mobile phase component can be measured through chromatographic response function, which is the resolution and asymmetry factor (As) (Snyder *et al.*, 1997). Resolution is one chromatographic response function that is widely used to measure the affinity between chromatogram peak. Separation between compounds is said to be perfect if the resolution obtained is more than 1.5 (Sastromahidjodjo, 2002). Asymmetry factor is a parameter to measure the sharpness of the peak produced in the chromatogram. According to Himawan (2011) an asymmetrical peak can produce inaccurate calculations, a decrease in the degree of resolution, and a peak exit time that is not reproducible. Symmetrical peaks are peaks that have As = 1, but the As value between 0.9-1.2 can still be said to optimum (Amina *et al.*, 2012).

In the research conducted, mobile phase optimization is done by selecting one of the mobile phases of 3 types of mixtures, A, B, and C. The selection of the first mobile phase is done by comparing the results of separation of compounds under 366 nm UV light. Internal selection is used in the mobile phase selection, which is the result of the extract of *P. niruri* endophytic fungi from PDB medium (Agastian *et al.*, 2013). Standard solution prepared at a concentration of 2000 ppm, then spotted the stationary phase. After drying, it is developed using all five mobile phase mixtures. The results of the development seen the separation under UV light 366 nm. From the results of elution, it can be seen that the best separation is obtained from the mobile phase C (chloroform: ethyl acetate: ammonium hydroxide = 5: 8: 0.005). This can be seen from the results of the stains of the alkaloid compounds produced with R_f 0.4 and the separation between the compounds that are optimum as in Figure 1.

After obtaining the best visual phase of mobile separation, the resolution and Asymmetry factor (As) were measured from the results of the densitogram. The measurement of the mobile phase C is compared with the mobile phases A and B. The results of the As calculation in the mobile phases A, B, and C are As for 5.3333; 0.8389; and 4.6667 in sequence. The As value obtained in each mobile phase is said to not meet the conditions that should be in the range 0.9 - 1.2. While the resolution measurement is obtained as in Table II.

Table II. Results of calculation of resolution

| Mobile phase | replication | peak | resolution | requirement | criteria |
|---|-------------|------|------------|-------------|----------|
| (A) chloroform:ethyl acetate:methanol (8:8:4) | 1 | 5 | 0.6000 | ≥ 1.5 | not |
| | | | 5.1430 | | yes |
| | 2 | 4 | 0.6000 | | not |
| | | | 4.5330 | | yes |
| | 3 | 6 | 0.8990 | | not |
| | | | 4.3478 | | yes |
| (B) chloroform:ethyl acetate: methanol: NH ₄ OH (8:8:4:0.005) | 1 | 8 | 1.3300 | ≥ 1.5 | not |
| | | | 1.3850 | | not |
| | 2 | 8 | 1.2900 | | not |
| | | | 1.4600 | | not |
| | 3 | 6 | 1.7500 | | yes |
| | | | 1.5500 | | yes |
| (C) chloroform:ethyl acetate:NH ₄ OH (8:8:0.005) | 1 | 2 | 0.6670 | ≥ 1.5 | not |
| | | | 3.2700 | | yes |
| | 2 | 5 | 0.5450 | | not |
| | | | 2.0000 | | yes |
| | 3 | 2 | 0.5000 | | not |
| | | | 2.7500 | | yes |

From the [Table II](#) above it can be seen that in the mobile phase chloroform:ethyl acetate:methanol: NH₄OH (8:8:4:0.005) the resolution criteria were met only on the third replication. In the mobile phase chloroform:ethyl acetate:methanol (8:8:4) the resolution criteria were met only on the right side of the peak alkaloid compound, as well as the mobile phase chloroform:ethyl acetate:NH₄OH (8:8:0.005). Although the results of the fulfillment of the resolution criteria and the movement phase chloroform:ethyl acetate:methanol (8:8:4) and chloroform:ethyl acetate:NH₄OH (8:8:0.005) were the same, the mobile phase chloroform: ethyl acetate: NH₄OH (8:8:0.005) was chosen because the KLT separation results were visually better under the lamp UV 366 nm ([Figure 1](#)).

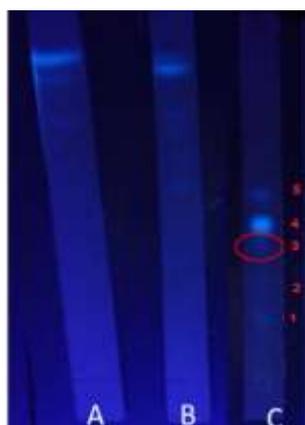


Figure 1. The results of the elution of the three mobile phase mixtures on UV lamp 366, mobile phase (A) : chloroform:ethyl acetate:methanol (8:8:4); (B) : chloroform:ethyl acetate:methanol: NH₄OH (8:8:4:0.005); (C) chloroform:ethyl acetate:NH₄OH (8:8:0.005). Stationary phase silica gel GF₂₅₄

Determination of the maximum wavelength

Measurements must be made at the maximum wavelength because the maximum wavelength of sensitivity is maximal so that the change in absorbance in each unit of concentration is greatest. In addition, at the maximum wavelength the shape of the absorbance curve is flat and complies with the Lambert-Beer law, and minimizes errors caused by re-installation of the wave length (Himawan, 2011). Densitogram at a wavelength of 200 nm are presented in Figure 2.

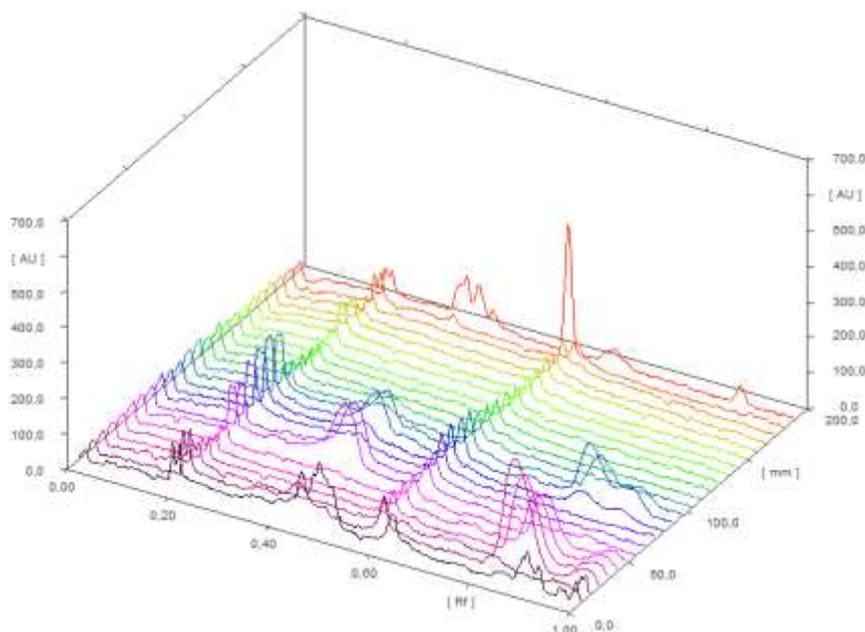


Figure 2. Densitogram at a wavelength of 200 nm

Determination of the maximum wavelength was carried out by measuring the standard solution and the other eleven samples at a concentration of 2000 ppm which was bottled on the TLC plate using Linomat 5 as much as 10 μ L. Development is carried out using the C mobile phase, then readings at a wavelength of 200–800 nm with a range of 50 nm. The densitogram results from 13 wavelengths obtained are then compared to the peak gain. The maximum wavelength is obtained from the densitogram with the highest peak at Rf of about 0.4 which is the Rf of the alkaloid compound. Based on the densitogram obtained, the best peak densitogram results are at the 200 nm wavelength as in Figure 2, so the maximum wavelength is set at 200 nm.

Linearity test

Linearity tests are carried out to measure abilities within a certain range in obtaining test results that are proportional to the amount of concentration of analytes in the sample. In this study, the linearity test was carried out using a standard solution made into 6 types of concentrations, namely 2000 ppm, 1800 ppm, 1600 ppm, 1400 ppm, 1200 ppm, and 1000 ppm. Measurements were made with one replication, then the AUC results and calculated concentrations were averaged. The average results of calculated concentrations and AUC are plotted with the average concentration as the x axis and the average AUC as the y axis, so that the calibration curve is obtained as shown in Figure 3.

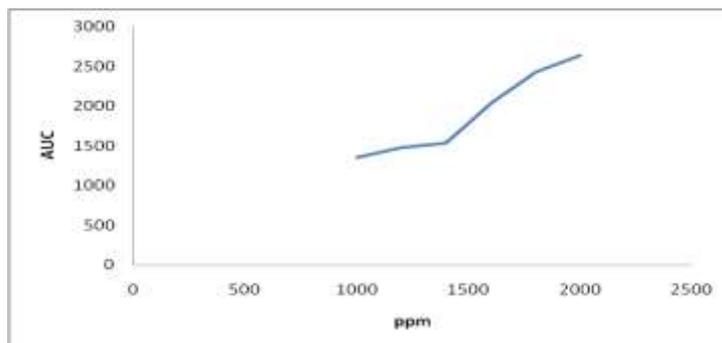


Figure 3. Calibration curve

Based on the results of the calibration curve, the equation $y = 10.278x - 83.023$, with R, slope, and intercept is 0.9769; 10,278; -83.023 in sequence. Based on the R results obtained, the linearity test can still be said to be fulfilling because the value of R is greater than the provisions of table R, with the requirements of table R being 0.9741 and the R linearity test obtained is 0.9769. (AOAC, 2014); (Hendarto *et al.*, 2018).

Accuracy and precision test result

Accuracy test is used to see the closeness between the received value as the reference value that has been received and the value obtained. In this study the reference value used is the standard solution of the PDB medium and the value obtained comes from the sample with various media treatments. Accuracy in a method can be determined by percentage recovery (Lories *et al.*, 2013).

In calculating accuracy, it is done by making 6 kinds of concentrations of fungerin solution as in the linearity test. Each solution was bottled in the stationary phase with three replications, then the recovery percentage was calculated from each concentration series. The calculation results show the results of percentage recovery 98.3233; 105.7880; 102.1342; 98.5954; 95.6018; and 107.4840 at a solution concentration of 1000 ppm, 1200 ppm, 1400 ppm, 1600 ppm, 1800 ppm, and 2000 ppm respectively. Based on the requirements of AOAC in 2014, the accuracy test meets the requirements if the recovery percentage is in the range of 90-110. From the calculation results it can be seen that the accuracy test meets the requirements at all concentrations.

The precision test aims to show the closeness between the measurement series obtained from several partial drawings from one sample that is the same as the predetermined conditions. Precision analytical procedures can be determined based on the percentage of RSD. In this study precision repeatability tests, namely precision with the same operational conditions in a short period of time.

The precision test was carried out by calculating % RSD from the series of solution concentrations of the solution that was replicated twice. From the calculation obtained % RSD data of 1.5159; 5.1523; 4.6228; 3.3842; 2.8042; and 4.8294 from a solution concentration of 1000 ppm, 1200 ppm, 1400 ppm, 1600 ppm, 1800 ppm, and 2000 ppm respectively. Based on the requirements set by AOAC in 2014, namely ≤ 7 , then from the calculation results at all concentrations meet the precision test.

Determination of the level of alkaloids in the sample

Carbon sources are used for normal growth fungi, especially glucose as an energy source to form primary metabolites. In fungi metabolism, carbon sources are an important component in Glutamine Synthetase (GS) which is the first step in the pathway for synthesis of important macromolecular compounds (Blankenship *et al.*, 2001). In the stationary phase, when glucose has been used up for the formation of secondary metabolites, fungi will use other more complex carbon sources (Kavanagh, 2005).

In its use, although glucose is the best carbon source for microbial growth, it is rarely used in the formation of secondary metabolites. Glucose can inhibit several syntheses of secondary

metabolites, one of which is ergot alkaloids. In the formation of secondary metabolites, for example in beta lactam, a fast-used carbon source (glucose, maltose, fructose) produces less beta lactam compared to slow used sugar (sucrose, galactose) (Drew and Demain, 1977).

Complex carbon sources such as starch in metabolism will be broken down into simpler sugars by the enzyme α -amylase to maltose to glucose. The glucose produced will experience glycolysis to pyruvate and enter the Krebs cycle. This oxaloacetate produced by the Krebs cycle is later converted into various amino acids which are precursors in the biosynthesis of alkaloid compounds.

In samples with carbon sources, the highest levels of alkaloid were obtained in starch samples followed by sucrose samples with amounts that were not much different (Figure 4). High levels of alkaloid in starch and sucrose samples can be obtained because both are slow used sugar with a more complex structure than other carbon sources used.

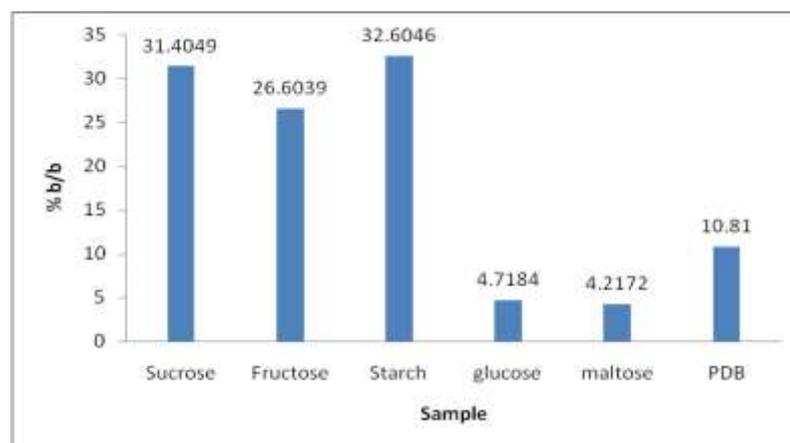


Figure 4. Diagram of carbon source samples

The pH condition of the medium is a factor for determining the metabolism and biosynthesis of secondary metabolites. This is because pH is related to the permeability of walls and cell membranes, so that it can affect the entry and exit of ions from medium nutrients (Merlin *et al.*, 2013).

In indole alkaloids namely slaframine and swainsonin produced by *Rhizoctonia leguminicola* derived from biosynthesis of pipekolat acid which is a derivative of L-lysine. In the biosynthesis of pipekolat acid it involves the enzyme saccharopine oxidase in sequence: L-lysine \rightarrow saccharopine \rightarrow 1-piperidein-6-carboxylic acid \rightarrow pipekolat acid. According to research that has been done, the enzyme saccharopine oxidase has the most optimum activity at pH 6 (Wickwire *et al.*, 1990).

For samples in various treatments the pH results of most alkaloids were obtained at pH 6. In the diagram results (Figure 5) showed an increase in the amount of alkaloid content at pH 6 compared to the PDB medium. This can occur due to the optimum activity of the enzyme saccharopine oxidase in the synthesis of alkaloids at pH 6. In addition, the growth of a good endophytic fungus will certainly affect the results of secondary metabolites. According to Ramadhani (2017) *Fusarium sp.* can grow well at pH 4.5-6. The optimum pH 6 condition for the growth of fungi and the production of the enzyme saccharopine oxidase which makes alkaloid levels in samples with a pH of 6 higher than the PDB medium. Sample diagram of pH treatment are shown in Figure 5.

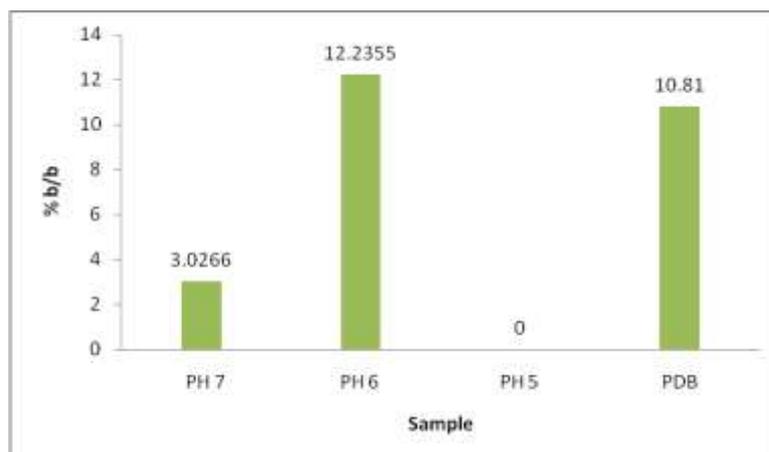


Figure 5. Sample diagram of pH treatment

Salinity is one of the environmental factors that can affect the results of endophytic secondary metabolites, because the habitat of the host endophytic fungi affects the salinity concentration needed. In endophytic fungi with hosts whose habitat is in the sea tends to require higher salinity compared to fungi whose hosts live in the soil (Mathan *et al.*, 2013). Along with increasing salinity levels, the cytoplasm in the cell wall will increase (Allakhverdiev *et al.*, 1999). The decreased intracellular salinity makes the condition become hypotonic so that water enters the plasma membrane. Conversely, in a hypertonic state with an increase in salinity levels, the water will come out of the plasma membrane so that the cell shrinks and the cell metabolites are inactive (Pratiwi, 2008). Diagram of salinity are shown at Figure 6.

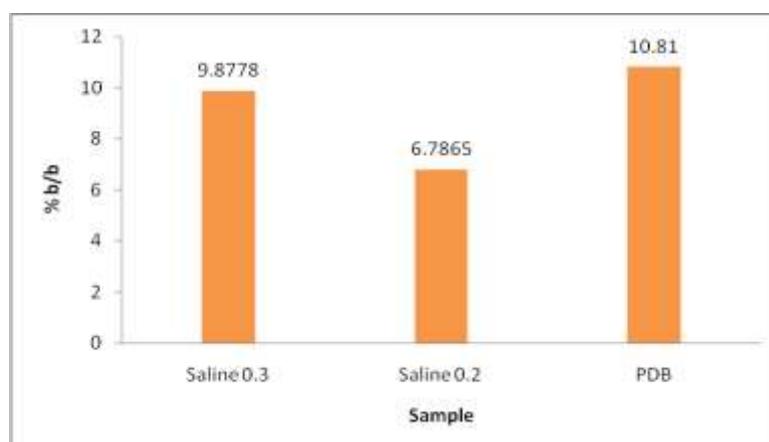


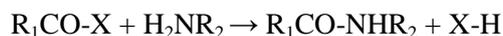
Figure 6. Diagram of salinity

In samples with differences in salinity concentration the highest alkaloid content was obtained at salinity of 0.3%. Compared with the acquisition of alkaloids from the PDB medium, the treatment of salinity samples that have been carried out produces fewer alkaloids (Figure 6). According to Merlin *et al.* (2013) the addition of concentrations of secondary metabolites continued to increase with increasing NaCl to a concentration of 3%, and began to decrease at a concentration of 3.5%. In this study the alkaloid levels in salinity samples were no more than PDB medium due to a lack of high salinity levels during fermentation which made endophytic fungi produce more nonpolar compounds (Rollando *et al.*, 2018a).

In addition to carbon sources, nitrogen sources are also used for cell growth at the beginning of fermentation (Rollando *et al.*, 2018b). At the end of cell growth, nitrogen is used to form secondary

metabolites through enzymatic reactions and metabolic processes (Stanbury and Whitaker, 1984). Nitrogen and carbon play an important role in glutamate synthesis which is used as the initial pathway for synthesis of macromolecular compounds in fungi (Kavanagh, 2005).

Almost all alkaloids are derivatives of amino acids. In alkaloid biosynthesis of amino acids using aliphatic nitrogen requires the formation of heterocyclic rings followed by the synthesis of new C-N bonds (Facchini *et al.*, 2001). Alkaloids can be produced by forming acid-amide bonds in the presence of carbonyl groups as in the reaction below (Rasmussen *et al.*, 2006).



The sample with the addition of a nitrogen source from $NaNO_3$ showed a decrease in the number of alkaloids obtained when compared to PDB media (Figure 7). Based on the reaction described above, there is no increase in alkaloid levels in nitrogen samples due to the absence of carbonyl groups which help to form acid-amide bonds, so that heterocyclic rings cannot form.

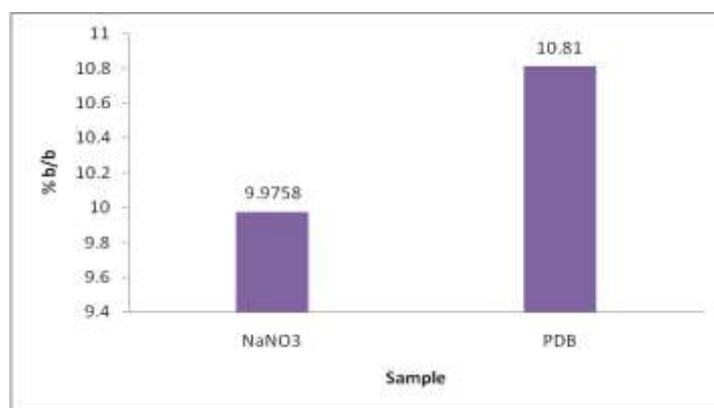


Figure 7. Diagram of a nitrogen source sample

CONCLUSION

TLC densitometry can be used as a method of measuring the alkaloid levels of extracts from fermented endophytic plants of valid meniran by fulfilling several requirements of validity parameters including linearity with $R = 0.977$, accuracy with % recovery between 90-100 in each concentration series, and % $RSD \leq 7$ in each concentration series. The results of measuring the alkaloid levels in the sample show that the good conditions for the culture of endophytic fungi of meniran to obtain maximum alkaloid levels are with carbon sources from starch and at pH 6 conditions.

ACKNOWLEDGEMENT

The authors are grateful to the Directorate General of Strengthening Research and Development, Ministry of Research, Technology and Higher Education of the Republic of Indonesia via the Higher Education Excellent Basic Research (PDUPT 2018, No. 3/E/KPT/ 2018 and No. 004/MACHUNG/LPPM/ SP2H-LIT/II/2018).

REFERENCES

- Agastian, P., J. N. Merlin, I. N. Christudas, and P. K. P. 2013. Optimation of growth and bioactive metabolite production :*Fusarium solani*, *Asian journal of pharmaceutical and clinical research*, 6:98-103.
- Allakhverdiev SI, Nishiyama Y, Suzuki I, Tasaka Y, Murata N.1999. Genetic engineering of the unsaturation of fatty acids inmembrane lipids alters the tolerance of Synechocystisto salt stress. *Proc Natl Acad Sci USA*96:5862–5867.

- Amina, M., Sharma P.R., Puri, S.C., Al-Youssef, H.M., Al-Taweel, A.M., Qazi, G.N. 2012. Effect of precursors feeding and manipulation on production of novel anticancer pro-drug camptothecin from endophytic fungus, *Brazilian Journal of Microbiology*, 43(4):1476-1489.
- Association of Official Analytical Chemists. 2014. 'A 2014 Overview', AOAC International.
- Blankenship, J.D., Spiering, M.J., Wilkinson, H.H., Fannin, F.F., Bush, L.P., Schardl, C.L. 2001, Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media, *Phytochemistry*, 58:395-401.
- Colpo, E., Vilanova, C.D.D.A., Pereira, R.P., Reetz, L.G.B., Oliveira, L., Farias, I.L.G., Boligon, A.A., Athayde, M.L., Rocha, J.B.T., 2014, Antioxidant effects of *Phyllanthus niruri* tea on healthy subjects, *Asian Pac. J. Trop. Med.* 7:113-118.
- de Souza, J.J., Vieira, I.J.C., Rodrigues-Filho, E., and Braz-Filho, R. 2011. Terpenoid from endophytic fungi, *Molecules*, 16:10604-10618.
- Drew, S.W. and Demain, A.L. 1977. Effect of primary metabolites on secondary metabolism, *Annu. Rev. Microbiol.*, 31:343-356.
- Facchini, P.J. 2001, Alkaloid biosynthesis in plants: Biochemistry, cell biology, molecular regulation, and metabolic engineering applications, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 52:29-66.
- Hendarto, G. C., Monica, E. and Rollando, R. 2018. Spectrophotometry UV-Vis Technique Combined with Chemometrics for Determination Phenylbutazone and Acetaminophen in Herbal Medicines?, *Journal of Global Pharma Technology*, 10(12 (Suppl.)):34-41.
- Himawan, D.E. 2011. Optimasi penetapan kadar kurkumin dalam sediaan cair obat herbal terstandar (OHT) merk "Kiranti[®]" dengan metode kromatografi lapis tipis (klt)-densitometri, *Skripsi*, S. Farm, Fakultas Farmasi, Universitas Sanata Dharma.
- Kavanagh, K. 2005. *Fungi: Biology and Applications*. John Wiley & Sons.56.
- Kumara, P.M., Soujanya, K.N., Ravikanth, G., Vasudeva, R., Ganeshaiah, K.N. and haanker, R.U. 2014. Rohitukine, a chromone alkaloid and a precursor of flavopiridol, is produced by endophytic fungi isolated from *Dysoxylum binectariferum* Hook.f and *Amoora rohituka* (Roxb), *Phytomedicine*, 21:541-546.
- Lories I.B, Mostafa, A.A and Girges, M.A. 2013. High performance liquid chromatography, TLC densitometry, first derivative and first-derivative ratio spectrophotometry for determination of rivaroxaban and its alkaline degradates in bulk powder and its tablets, *Journal of Chromatography and Separation Techniques*, 4(8):1-6.
- Luckner, M., 1984. *Secondary metabolism in microorganism, plants, and animals*, 2nd Edition, Springer-Verlag Berlin Heidelberg.
- Mathan, S., Subramanian, V., dan Nagamony, S. 2013. Optimization and antimicrobial metabolite production from endophytic fungi *Athelia rolfsii* KC 582297, *European Journal of Experimental Biology*, 3: 138-144.
- Merlin, J.N. Agastian, P.J, I.V.S. Nimal Christudas, and Praveen Kumar. 2013, Optimization of growth and bioactive metabolite production : *Fusarium solani*, *Asian journal of pharmaceutical and clinical research*, 6:98-103.
- Murphy, B. R., Doohan, F. M., and Hodkinson, T. R. 2015. Fungal root endophytes of a wild barley species increase yield in a nutrient-stressed barley cultivar. *Symbiosis*, 65: 1-7.
- Okoli, C. O., Ibiam, A. F., Ezike, A. C., Akah, P. A. and Okoye, T. C. 2010. Evaluation of antidiabetic potentials of *Phyllanthus niruri* in alloxan diabetic rats, *Afr J Biotech-nol*, 9: 248-259.
- Pratiwi, S.T., 2008. *Mikrobiologi Farmasi*, Erlangga Medical Series.
- Ramadhani, S.H. 2017. Isolasi dan identifikasi jamur endofit pada daun jambang (*Syzigium cumini* L), *Jurnal Ilmiah Mahasiswa Fakultas Keguruan dan Ilmu Pendidikan Unsyiah*, 2(2):77-90.
- Rasmussen, S., Parsons, A.J, Bassett, S., Christensen, M.J, Hume, D.E, Johnson, L.J, Johnson, R.D, Simpson, W.R, Stacke, C., Voisey, C.R, Xue, H, Newman, J.A. 2006. High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*, *New Phytologist*, 173:787-797.
- Rodriguez, R.J., White, Jr.J.F., Arnold, A.E. and Redman, R.S. 2009. Fungal endophytes: diversity and functional roles, *New Phytology*, 182: 314-330.

- Rollando R, Sitepu R, and Monica E. 2018a. Cytotoxic activity of 2- iminoethyl 2-(2-(1-hydroxypentan-2-yl) phenyl) acetate from sterculia quadrifida R.Br ethyl acetate fraction. *Journal of Global Pharma Technology*. 10 (06), 204-212.
- Rollando, Aditya, M. Notario, D., Monica, E, Sitepu, R,. 2017. Kajian aktivitas antibakteri, antioksidan, dan sitotoksik fungi endofit genus *Fusarium sp.* isolat daun meniran (*P. niruri* Linn.), *Pharmaciana*, 7:95-104.
- Rollando, R., Prilianti, K.R. 2018. Sterculia quadrifida R.Br ethyl acetate fraction increases cisplatin cytotoxicity on T47D breast cancer cell. *International Journal of Pharmaceutical Research*.10(03): 204-212.
- Rollando R. 2018. Combination of hedyotis corymbosa L. and tinospora crispa ethanolic extract increase cisplatin cytotoxicity on T47D breast cancer cells. *Asian J Pharm Clin Res*, 11 (7), 171-177.
- Rollando, R., Engracia, M., Irawati, L.N., Hartono, E, Monica, E., Sitepu, R. 2018b. Isolation of active compound from Zingiber Purpureum Roxb. Using bioassay guided fractionation method for WIDR colon adenocarcinoma cell line. *Journal of Global Pharma Technology*.; 10(07):54-60.
- Sastrohamidjojo, H., 2002. Spektroskopi, Penerbit Liberty , Yogyakarta, 11, 22-32.
- Selim, K. A., El-Beih, A. A., Abdel-Rahman, T. M., dan El-Diwany, A. I. 2012. Biology of endophytic fungi, *Curr. Res. Environ. Appl. Mycol*, 2(1):31-38.
- Sherma, J. and Fried B. 2003. *Handbook of Thin-Layer Chromatography*, 3rd Edition, Marcel Dekker, Inc., New York, America.
- Smolianiuk, E.V, Bilanrenko, E.N, Tereshina, V.M, Kachalkin, A.V, Kamzolkina, O.V. 2013. Effect of sodium chloride concentration in the medium on the composition of the membrane lipids and carbohydrates in the cytosol of the fungus *Fusarium sp.*, *Microbiology*, 82(5):600-608.
- Stanbury, P.F. and Whitaker, A., 1984. *Principles of Fermentation Technology*, 1st Ed. edition. ed. Pergamon, Oxford Oxfordshire, New York. 239.
- Synder, L. R., Kirkland, J. J., Glajh, J., L., 1997. *Practical HPLC Method Development*, 2nded, John Willey Sons, Inc., New York, pp.211, 236.
- Tang, Z., Rao, L., Peng, G., Zhou, M, Shi, G., and Liang, Y. 2011. Effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*, *journal of Medicinal Plants Research*, 5(11):2192-2200.
- Vrancheva, R. Ivanov, I., Marchev, A., Pavlov, A. 2012. Qualitative and Quantitative Determination of Protopine in *Fumaria spp.* By TLC Densitometry Method, *J. Bio Sci. Biotech.*, 1(3):255-259.
- Wickwire, B.M, Harris, C.M, Harris, T.M, Broquist, H.P. 1990. Pipecolic acid biosynthesis in *Rhizoctonia leguminicola*, *The Journal of Biological Chemistry*, 265(25):14748-14753.
- Yuniati Y, Rollando R. 2018. Isolation of antibacterial compounds from endophyte fungal of fusarium sp. In *Phyllanthus niruri* Linn. Leaves. *J Pharm Sci & Res*, 10:260-264.
- Yuniati, Y., Yuliati, L., Monica, E., Rollando, R. 2018. Effect of variation conditions fermentation to production biomass of endophytic fungi *Athelia rolfsii* strain orchid. *J Pharm Sci & Res*, 10: 2862-2865.
- Yuniati Y, Alfanaar R, Rollando R. 2018. Structural conformational study of isoflavon derivatives in soybean using semiempirical methods. *Journal of Global Pharma Technology*. 10 (05): 220-225.
- Zhao, J., Shan, T., Mou, Y. and Zhou, L. 2011. Plant derived bioactive compounds produced by endophytic fungi, *Mini reviews in medicinal chemistry*, 11: 159-168.
- Zum Felde, A., Mendoza, A., Cabrera, J.A., Kurtz, A., Schouten, A., Pocasangre, L., & Sikora, R. A. 2009, The burrowing nematode of banana: Strategies for controlling the uncontrollable. In Jones, D. & Van den Bergh, I. (Eds.), *Proceeding of International Symposium on Banana Crop Prot. Sust. Prod. & Impr. Livelihood*. *Acta Horticulturae*, 828:101-108.