Research Article

Optimation of Combination of N-Hexane Solution and Ethyle Acetate on Secondary Metabolite Compounds Profile of Streptomyces hygroscopicus

Optimasi Kombinasi Pelarut N-Hexane dan Ethyl Acetate dari Profil Senyawa Metabolit Sekunder Streptomyces hygroscopicus

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ABSTRACT

Streptomyces hygroscopicus (S.hygroscopicus) is a Gram-positive soil bacterium that can produce secondary metabolites from fermentation that have a therapeutic effect. The fermented S. hygrocospicus metabolites that are still in the form of crude extracts are difficult to develop as drug preparations because the active compounds are not yet known, so it will be challenging to determine the dosage of drugs that have a therapeutic effect. Therefore, it is necessary to carry out exploratory research to narrow down the secondary metabolite profile from the fermentation of S. hygroscopicus, using extraction and fractionation methods, which are then identified by Thin-Layer Chromatography (TLC) using a combination of solvents. This study used the extraction method with a separating funnel. The fractionation was carried out using the BUCHI (Sepacore®) Flash Chromatography and Reveleris® PREP Purification System column chromatography gradually using ethyl acetate and n-hexana. 47 and 60 of the fractionation results were taken as samples, that further were profiled using TLC and given the appearance of 10% KOH stains and p-Anisaldehyde - sulfuric acid, so that various classes of compounds with different Rf values were obtained, namely Monoterpenes, Triterpenes, Steroids, Saponins, Coumarin, Scopoletin, and Alkaloids.

Keywords: Extraction, flash chromatography, fractionation, thin layer chromatography, Streptomyces hygroscopicus

ABSTRAK

Streptomyces hygroscopicus (S. hygroscopicus) merupakan bakteri tanah Gram positif yang dapat menghasilkan metabolit sekunder hasil fermentasi yang memiliki efek terapeutik. Metabolit S. hygrocospicus hasil fermentasi yang masih berupa ekstrak kasar sulit untuk dikembangkan sebagai sediaan obat karena senyawa aktifnya belum diketahui, sehingga akan sulit untuk menentukan dosis obat yang memiliki efek terapeutik. Oleh karena itu, perlu dilakukan penelitian eksplorasi untuk mempersempit profil metabolit sekunder hasil fermentasi S. hygroscopicus, menggunakan metode ekstraksi dan fraksinasi, yang kemudian diidentifikasi dengan Kromatografi Lapis Tipis (KLT) menggunakan kombinasi pelarut. Penelitian ini menggunakan metode ekstraksi dengan corong pisah. Fraksinasi dilakukan menggunakan BUCHI (Sepacore®) Flash Chromatography dan kromatografi kolom Reveleris® PREP Purification System secara bertahap menggunakan etil asetat dan n-heksana. 47 dan 60 dari hasil fraksinasi diambil sebagai sampel, yang selanjutnya diprofilkan menggunakan KLT dan diberi kenampakan noda KOH 10% dan p-Anisaldehida - asam sulfat, sehingga diperoleh berbagai golongan senyawa dengan nilai *Retardation Factor* (Rf) berbeda yaitu Monoterpenes, Triterpen, Steroid, Saponin, Coumarin, Scopoletin, dan Alkaloid.

Kata Kunci: Ekstraksi, flash chromatography, fraksinasi, kromatografi lapis tipis, *Streptomyces hygroscopicus*

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INTRODUCTION

Streptomyces hygroscopicus (*S.hygroscopicus)* is a Gram-positive group of soil bacteria that can form filaments similar to fungi. The genus *Streptomyces* is widely known for its ability to produce secondary metabolites from fermentation, which can act as antibiotics, antifungal, antiviral, antitumor, and immunosuppressive (1). The secondary metabolites of *S.hygroscopicus* have also been found to contain eponemycin and rapamycin analogs, which have antibiotic and antimalarial activities (2).

There has been an extensive number of studies related to the use of *S. hygroscopicus* as candidates for *Active Pharmaceutical Ingredient (API)* in the pharmaceutical research (2-7). The results of the studies mainly indicated that there is a fermentation phase that produces proteins, lipopeptides, peptidoglycan, and secondary metabolites that have the potential capability to be used as drug candidat (2). Based on the results of the literature study, it is known that the secondary metabolites of *S. hygroscopicus* extracted using several solvents can serve various activities, for example, antimalarial and antibacterial activity from ethyl acetate extraction (4), antibacterial activity from ethanol extraction, and antitumor activity from n-hexane extraction (8).

Streptomyces hygroscopicus fermented metabolite that is still in crude extract form is difficult to be used in determining drug dosage to provide therapeutic effects since the active compound is still unknown (9). Therefore, research to discover the isolate of secondary metabolites from the crude extract is necessary. First, there is a need to narrow the profile of metabolic compounds using extraction with many types of solvents (9,10). Secondly, it is important to group them based on their metabolite groups as an initial prediction of bioactive abilities. For example, compounds from the flavonoid group and glycosides generally have antibacterial activity through their ability to inhibit the synthesis of nucleic acid and function of the cytoplasmic membrane (11), while the derivatives of epoxomicin and eponemycin have antimalarial activity by inhibiting the complex *proteasome* (4).

The extraction method uses the solubility principle *like dissolve like*, namely polar solvents, which will dissolve polar compounds, while conversely, non-polar solvents will dissolve non-polar compounds. Ethyl acetate solvent was chosen because it is semi-polar, which is volatile and can dissolve compound from semi-polar to non-polar compounds in bacterial cell walls (Harborne, 1987) and it has been shown the extractions from a semi-polar solvent can produce compounds that have antimalarial activity from *S. hygroscopicus* (2).

The continued step after the extraction process for the separation of compounds is fractionating *S.hygroscopicus* based on the degree of polarity with ethyl acetate and n-hexane solvents. Both solvents are used because, based on literature studies, most secondary metabolites that have antimalarial effects are commonly distributed in semi-polar although tend to be non-polar properties. The polarity of ethyl acetate is semi-polar, and n-hexane is non-polar. The fractionation was carried out with these two solvent mixtures to obtain all secondary metabolites from polar to non-polar (10,11). The compound groups contained within the fraction results can be further identified using the Thin Layer Chromatography (TLC). This study aims to optimized eluent variations used for compound separation and to identify the compound groups using TLC method.

METHOD

Research Design

The research was conducted using a descriptive exploratory research design in the Laboratory of Pharmacy Faculty of Medicine Universitas Brawijaya. *S. hygroscopicus* was obtained from LIPI Microbial Collection Cibinong and sub-cultured in the Laboratory of Microbiology Faculty of Medicine Universitas Brawijaya. The crude extract of secondary metabolite of *S. hygroscopicus* was made using previously published method and the active antimalaria effect of this crude extract was tested before as described in previous article (data not shown here) (4). The fractions derived from the crude extract of secondary metabolite extract of *S. hygroscopicus* were then explored. The fractionation process was performed using Sepacore® Flash Chromatography and Reveleris® PREP Purification System Chromatography with gradient ethyl acetate and n-hexane solvents. The results of these fractions were validated using TLC to determine ethyl acetate: n-hexane eluent combination as well as to analyze the existing metabolite compounds. We decided the ethyl acetate: n-hexane volume combination as eluent and type of spraying reagents in the TLC procedure for independent variable. Furthermore, we checked for the spot characteristics appeared at the TLC plate such as Retardation factor (Rf) score and spot color after spraying as dependent variable. These TLC spot characteristics were then matched with database for compound group prediction.

Method of Fractionation I

The crude extract of secondary metabolite of *S. hygroscopicus* which obtained as previously described (4) was fractionated using Buchi Sepacore® Flash Chromatography with gradient and isocratic eluent systems. This procedure was performed in the Laboratory of Pharmacognosy and phytochemicals, Faculty of Pharmacy Universitas Airlangga under the expert jugdement. First, the gradient system started from ethyl acetate 100% n-hexane 0% to ethyl acetate 0% n-hexane 100% for 100 minutes, followed by 100% ethyl acetate isocratic eluent system for 20 minutes. The flushing process was then carried out with a gradient system of ethyl acetate 100% methanol 0% to ethyl acetate 0% methanol 100% for 30 minutes and followed by isocratic methanol for 20 minutes.

Method of Fractionation II

The results of previous fractionation process were replicated by fractionation using the Reveleris® PREP Purification System in the. The details are as follows; the gradient mode was set between n-hexane 100% and 100% ethyl acetate for 70 minutes with a flow rate of 5mL / minute. This was continued with 100% isocratic mode with ethyl acetate for 15 minutes, followed by flushing using methanol to push the remaining compounds on the tool. The details on fractionation for 101 minutes are as follows: gradient mode was between 100% n-hexane and ethyl acetate 100% for 70 minutes with a flow rate of 5mL / minute. After that, 100% isocratic mode was used with ethyl acetate for 10 minutes and followed by flushing by methanol to push the remaining compounds in the tool.

Thin Layer Chromatography (TLC)

The fractionation I and II results were then evaporated into crystals and dissolved with 1 mL methanol. Then 20 μl were dotted on the TLC plate (silica GF stationary phase size 20 cm x 20 cm x 1 mm), and dried. Subsequently, several mobile phases were used to eluate the samples. Filter paper in the CAMAG™ chamber containing the mobile phase solution was a marker of saturation. After saturated, the TLC plate is inserted into the chamber. After the elution was complete, the TLC plates were dried. The results were marked and observed under CAMAG™ UV lamp λ 254 and 366 nm. The p-Anisaldehyde - Sulfuric Acid 10% staining with 120^{0}C heating as well as 10% KOH staining were used as stain-appearing reagents on the CAMAG™ UV lamp λ 254 and 366 nm to see compounds whose previous TLC stains were not visible.

RESULTS

Results obtained from fractionation were TLC spots of 47 fractions from fractionation I (Sepacore® Flash Chromatography) and 60 fractions from fractionation II (Reveleris® PREP Purification System Chromatography). Fractionation I resulted in fractions 1 – 47. TLC results of fraction 1 – 47 that sprayed with p-Anisaldehyde - sulfuric acid and heated at 110$^°$C for 5 minutes are shown in Figure 1. The results showed spots in fractions 11, 12, 13, 14, and 15 with Rf values of 0.24 and 0.41 when irradiated with UV λ 254 and 366 nm. These spots are considered monoterpenes, triterpenes, or steroids because they appeared as gray or purple fluorescence under UV λ 254 nm and blue fluorescence under UV λ 366 nm (12). Besides that, the results of observations on UV light λ 254 nm revealed dark gray spots on fractions 33, 35, 36, and 39. However, when illuminated with UV λ 366 nm, it was found bright blue spots on fractions 33 and 39 with Rf values of 0,28, and 0.7. These results were then compared to the literature (12). It could be concluded that the fractions 33, 35, 36, and 39 were monoterpenes, triterpenes, or steroids because of the similar characteristics of spots and stain with those in the literature. The TLC results of fraction 41 – 47 under UV light λ 254 nm did not get any spots. Meanwhile, when irradiated with UV λ 366 nm, a bright blue spot was observed at fraction 42 with Rf values 0.3 and 0.7. These spots are then considered a saponin compound group due to the blue fluorescence under UV λ 366 nm after p-Anisaldehyde - sulfuric acid spraying (12).

Results of all 47 fractions are then summarized in Table 1 with details of Rf values 0.24 and 0.41 in fractions 11, 12, 13, 14, and 15; Rf 0.28 and 0.7 in fractions 33, 35, 36 and 39; and Rf values 0.29 and 0.7 in fraction 42. These values were obtained from the observation of the TLC plate that had been sprayed with p-Anisaldehyde - sulfuric acid and heated 110$^°$C for 5 minutes under 254 and 366 nm UV light. Fractionation II yielded 60 fractions (1 – 60). The TLC results of fractions 1 – 20 showed no spots before and after 10% KOH and p-Anisaldehyde - sulfuric acid spraying under UV light λ 254 and 366 nm. The results of TLC at fractions 21-40 sprayed by 10% KOH stain are presented in Figure 2. The TLC plate was then observed in UV light λ 254 nm and UV λ 366 nm. The observations on UV light λ 254 nm showed weak dark gray spots on fractions 36, 37, 38, and 39 with an Rf value of 0.24. A different result was found on observations with UV light λ 366 nm where blue spots appeared on fractions 36, 37, 38, and 39 with an Rf value of 0.24. These results were then compared to the literature (12,13). It is stated that compounds derived from coumarin, scopoletin, or alkaloids sprayed with 10% KOH will produce a blue/blue-green color when exposed to UV light λ 366 nm and dark gray spots at UV λ 254 nm. Therefore, it could be concluded that the fractions 36, 37, 38, and 39 were derivatives of coumarin, scopoletin, or alkaloids, due to dark spots on UV λ 254 nm and blue stain when irradiated with UV λ 366 nm.

Table 1. Results of TLC fractionation I (first) ethyl acetate: N-Hexane *Streptomyces hygroscopicus* with (Sepacore®) flash chromatography

| Sample	Motion Phase	Comparison in (10ml)	Rf Value	Extracted Metabolites
Fractions 11, 12, 13, 14, and 15 (p-Anisaldehyde - sulfuric acid)	Ethyl Acetate: N-Hexane	1 : 1	0.24 and 0.41	Monoterpenes, Triterpenes, Steroids
Fraction 33, 35, 36, 39 (p-Anisaldehyde - sulfuric acid)	Ethyl Acetate: N-Hexane	1 : 1	0.28 and 0.7	Monoterpenes, Triterpenes, Steroid
Fraction 42 (p-Anisaldehyde - sulfuric acid)	Ethyl Acetate: N-Hexane	1 : 1	0.29 and 0.7	Saponins
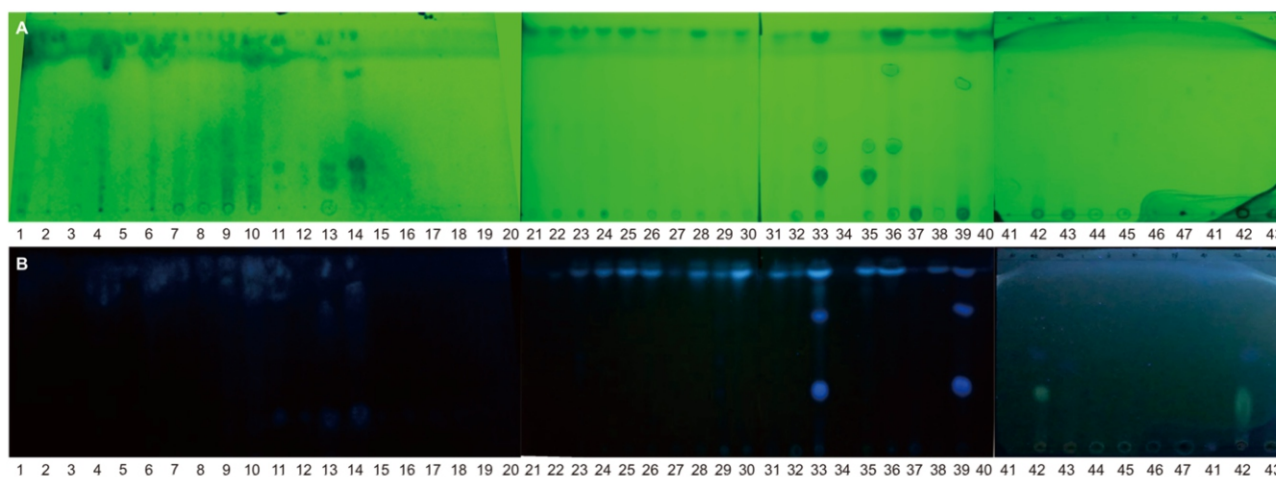
Figure 1. TLC fraction 1 – 47 from fractionation I used p-Anisaldehyde - sulfuric acid staining 254 nm (A) & 366 nM (B) UV light
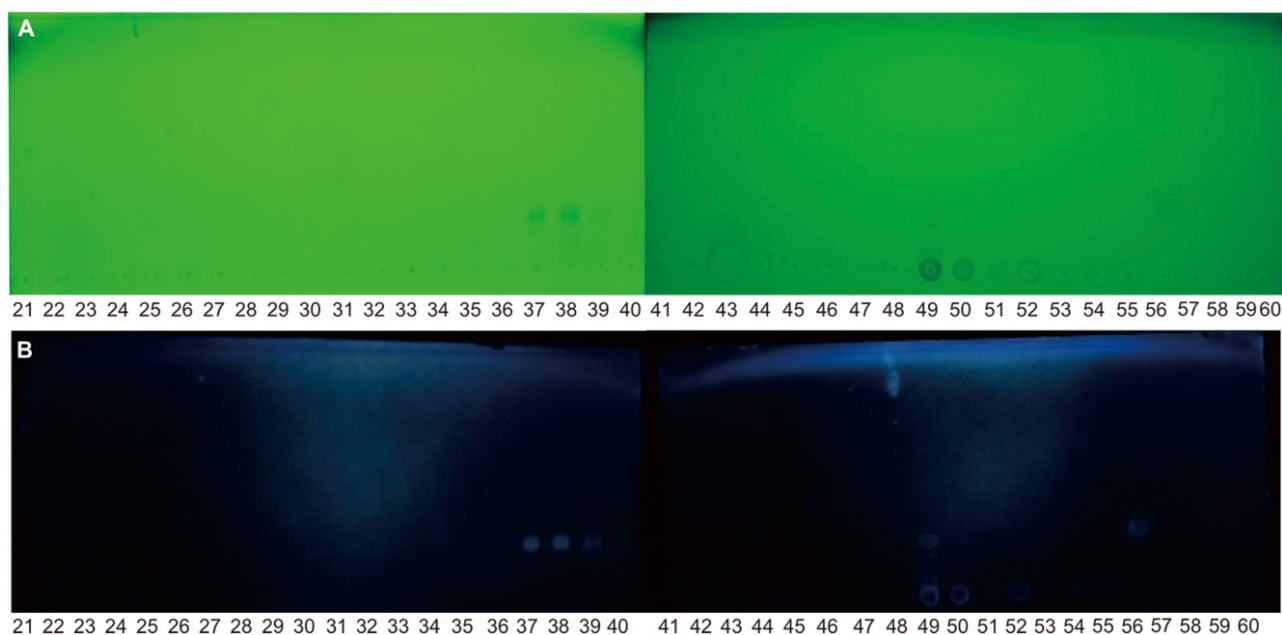
Figure 2. TLC Fraction 21-60 from fractionation II using 10% KOH staining under 254 nm (A) & 366 nm (B) UV light

Furthermore, the TLC results of fraction 41 – 60 observations under UV light λ 254 nm showed no spots after 10% KOH spraying (Figure 2). However, on observations with UV light λ 366 nm, it was found that a weak blue stain was found at fraction 49 and 56 with an Rf value of 0.42. These results were then compared to the literature (12,13), stating that compounds derived from coumarin, scopoletin, or alkaloids sprayed with 10% KOH will produce blue or greenish-blue color when exposed to UV light λ 366 nm. So, it could be concluded that the fractions 49 and 56 were derivatives of coumarin, scopoletin, or alkaloids due to the presence of blue spots when irradiated with UV λ 366 nm.

TLC results in fraction 21-40 after being sprayed with p-Anisaldehyde - sulfuric acid and heated to 110°C for 5 minutes are shown in Figure 3. The results showed spots on fractions 36, 37, 38, and 39 with an Rf value of 0.24 when exposed to UV light λ 366 nm. These results then compared to the literature (12), and it was known that the spots of the saponin group compounds will only show blue or purplish-blue spots at UV λ 366 nm when the TLC plate has been sprayed with p-Anisaldehyde - sulfuric acid. So that it could be concluded that the fractions 37 and 38 were saponins due to the presence of blue spots in those fractions when irradiated with UV λ 366 nm. Furthermore, the TLC results of fraction 41 – 60 under UV light λ 254 nm showed gray spots in fractions 49, 50, 51, 53, 54, 55, 56, and 57 with an Rf value of 0.42. But, fraction 52 showed a gray spot with an Rf
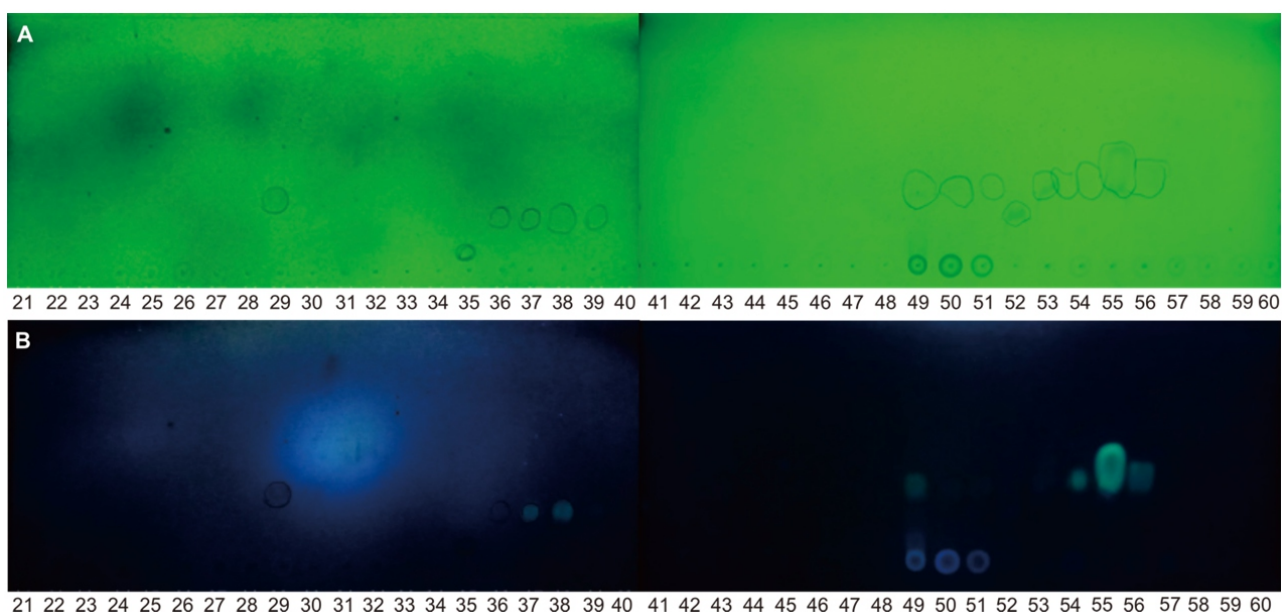

Figure 3. TLC Fraction 21-60 fractionation II using p-Anisaldehyde - sulfuric acid staining under 254 nm (A) & 366 nm (B) UV light

value of 0.4. Then, the TLC plate was irradiated with UV λ 366 nm, and it was found bright blue stains on fractions 49, 50, 51, 53, 54, 55, 56, and 57 with an Rf value of 0.42, but fraction 52 showed a blue stain with Rf value 0.4.

Based on the literature (12), it was known that when the TLC plate has been sprayed with p-Anisaldehyde - sulfuric acid, the spots of the monoterpenes, triterpenes, and steroids group will show gray spots under UV λ 254 nm and blue spots under UV λ 366 nm. Therefore, it is possible that the fractions 49, 50, 51, 52, 53, 54, 55, 56, and 57 were monoterpenes, triterpenes, steroids, and saponins group due to the presence of gray spots when exposed to UV λ 254 nm and blue color when irradiated with UV λ 366 nm. The Rf values of all 60 fractions are summarized in Table 2 below.

Table 2. Analysis results of TLC fractionation II (second) of Ethyl Acetate: N-Hexane *Streptomyces hygroscopicus* with Reveleris® PREP purification system

Sample	Motion Phase	Comparison in (10ml)	Rf Value	Extracted Metabolites
Fractions 36, 37, 38, and 39 (KOH 10%)	Ethyl Acetate: N-Hexane	1: 1	0.24	Coumarin, Scopoletin, and Alkaloid
Fractions 37 and 38 (p-Anisaldehyde - sulfuric acid)	Ethyl Acetate: N-Hexane	1: 1	0.24	Saponin
Fraction 49 and 56 (KOH 10%)	Ethyl Acetate: N-Hexane	1: 1	0.42	Coumarin, Scopoletin, and Alkaloid
Fractions 49, 50, 51, 52, 53, 54, 55, and 56 (p-Anisaldehyde - sulfuric acid)	Ethyl Acetate: N-Hexane	1: 1	0.42 and 0.4	Monoterpenes, Triterpenes, and Steroids

DISCUSSION

Extraction preparation was carried out in 3 types of extraction, with each extraction using a solvent for each polarity, namely: ethanol, ethyl acetate, and n-hexane. Ethanol was chosen because it is a universal solvent and is polar in nature, which can almost attract all the chemical contents in the plants. Even it can extract the intracellular or essential extracellular components located in bacterial cells. Ethyl acetate is an intermediate polar solvent that is volatile, non-toxic, non-hygroscopic, and selective in dissolving polar and non-polar compounds. The choice of n-hexane as a solvent was due to its stable and volatile characteristics. It is selective in dissolving non-polar compounds, relatively cost-effective compared to other non-polar solvents, such as petroleum ether, and has a lower toxicity than chloroform. The liquid extraction employed *like dissolve like* separation-based principle. Polar solvents will dissolve polar compounds, and non-polar solvents will dissolve non-polar compounds. After those extractions, two phases will be formed after the addition of the solvent (14).

In this study, the spots in the TLC plate appeared under 254 and 366 nm UV radiation. It used 10% KOH and *p-Anisaldehyde - sulfuric acid* as a stain. The disadvantage of UV lamps as an identifier is some spots may be invisible under the UV or visible light; thus, it makes some difficulties in Rf and Rs determination. This highlights the need for a proper stain-appearing reagent to reveal

previously invisible spots or to enhance the visibility of existing spots. The selection of this stain-appearing reagent was based on the chemical compounds contained within the sample, which was carried out by phytochemical screening using universal stains and/or based on the previous research.

TLC analysis is an initial method aimed to determine the type of compounds within the crude extract. TLC also is aimed to determine the most optimal eluent type and comparison in the initial fractionation process of *(Sepacore®) Flash Chromatography* and Reveleris® PREP Purification System. The eluent was used with a variety of solvent ratios ranging from low to high polarity. Eluent is said to be optimal when it can completely separate the spots in the TLC results. The optimal eluent used in this study was the n-hexane: ethyl acetate (1: 1) eluent for all extracts. The stationary phase was a silica G60 PF 254 TLC plate under the 254-366 nm UV lamp.

The three solvents of ethanol, ethyl acetate, and n-hexane gave different Rf results. Ethanolic extract revealed no spots, which is probably due to inappropriate TLC eluent selection. However, the TLC result of ethyl acetate and n-hexane extract revealed spots with Rf values of 0.21 & 0.78, respectively (Figure 5.2). Compared with the results of the previous study conducted by Rivo et al. (2013), the ethyl acetate extract in this TLC profile belongs to *Streptomyces hygroscopycus* and had a spot with an Rf value of 0.76 (ethyl acetate: n-hexane= 4: 1 (v/v) eluent). The importance of determining the eluent is it functions as an initial reference in the further separation process (4).

The optimization TLC results showed most spots were produced from ethyl acetate and n-hexane solvents. It can be assumed that most secondary metabolites were semi-polar to non-polar. The previous studies also show that secondary metabolites with antimalarial activity were obtained from semi-polar and non-polar solvents (4,8,15). Optimal TLC screening results should be able to separate compounds with a minimum Rf value difference of 0.1.

These initial results then became a reference for determining the solvent used in the further advanced fractionation stage. The purpose of fractionation is to separate compounds based on polarity using two solvents with different polarity levels. The different levels of polarity affect the type of compound that can be extracted (16,17). Fractionation I (Sepacore® Flash Chromatography) was performed using TLC with 1: 1 (v / v) ethyl acetate: n-hexane eluent and sprayed with p-anisaldehyde – sulfuric acid stains appearing agent to determine the class of compounds. The TLC results of fractionation I exhibited the compound groups of *Monoterpenes, Triterpenes,* Steroids, and Saponin. *Monoterpenes, triterpenes,* and steroids were at positions 11, 12, 13, 14, and 15. The spots had Rf values of 0.24 and 0.41 and appeared to have a dark color in 254 nm UV light and blue color on 366 nm UV light. Furthermore, *monoterpenes, triterpenes,* and other steroids appeared at positions 33, 35, 36, and 39. The spots had Rf values of 0.28 and 0.7 with a dark color on 254 nm UV light and blue color on 366 nm UV light.

Meanwhile, position number 42 showed spots with Rf values of 0.29 and 0.7 and purple-blue stained under 366 nm UV light, which indicates the alkaloid or saponin compounds. The results of previous studies were replicated again in fractionation II using the *Reveleris® PREP Purification System*. *Monoterpenes, triterpenes,* and

steroids groups in fractionation II were shown at position number 49, 50, 51, 52, 53, 54, 55, and 56. The spots had Rf values of 0.4 and 0.42 with black color under 254 nm UV light and blue color under 366 nm UV light. In addition, metabolite derivatives of *Coumarin, Scopoletin, and Alkaloid* were found at position number 36, 37, 38, 39, 49, and 56. The spots had Rf 0.24 and 0.42 with black color under 254 nm UV light and a greenish-blue color under 366 nm UV light. Then, alkaloid or saponin compounds appeared at position number 37 and 38 with a spot characterized by an Rf value of 0.24 and purple-blue color under 366 nm UV light.

The TLC results showed an inconsistency between fractionation 1 *(Sepacore®) Flash Chromatography)* and fractionation 2 (*Reveleris® PREP Purification System*), where the spot positions with the same Rf value were obtained in different fractions. This result is unfavorable because if the spot characteristics that need to be isolated are not consistent, it will be difficult to replicate it in the future. The cause of this inconsistency may be due to the separation process, which uses peak spectrum-based separation while the extract is still crude. The peak spectrum-based separation should only be used if the extract has been through a purification process repeatedly before. This unsuitable result may also be due to the different detectors used. Fractionation II (Reveleris® PREP Purification System) detects peaks using UV-visible and ELSD as detector while fractionation I (Sepacore® Flash Chromatography) separates peaks that appear on the UV-visible detector only.

The difference in the flow rate is assumed to be the most crucial reason why the number of fractions produced is different. Fractionation II uses a flow rate of 5 mL/minute while fractionation I uses a flow rate of 10 mL/minute. This difference will significantly affect the interaction time between the stationary phase of the column and the compounds contained in the extract so that it may produce chromatogram profiles with different retention times (18).

Another major difference may be caused by the different types of content columns used. The fractionation I used Silica Gel 60 with a specification of pore size of 60 Å, a particle size of 40-63 μm, an irregular shape, with an area of 500 m^2/g as much as 33 grams. In contrast, fractionation II used FlashPure ID Silica with pore size specification of 53-80 Å, a particle size of 35-45 μm, an irregular shape, with an area of 500-600 m^2/g. The difference in the silica particle size will affect the column efficiency and increases the amount of silica gel in the column. The pore size will affect the column area where the smaller the pore size between particles will increase the area that can interact with compound molecules. However, if the pore size is too small, it can reduce the efficiency and resolution of large molecules (18).

To conclude, there were 13 spots on the TLC result of fractionation I and 14 spots on TLC results of fractionation II. These spots are Monoterpenes, Triterpenes, Steroids, Saponins, Coumarin, Scopoletin, and Alkaloid compound group. Several factors that determine the result of fractionation include flow rate and column used. These results may be used for further purification process utilizing conventional column chromatography or other advanced instruments.

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