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Antibacterial activity of *Plantago major* leaves against *Streptococcus pyogenes* ATCC 19615 as a cause of tonsilitis

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Abstract. *Astuti AD, Etikawati N, Pangastuti A. 2020. Antibacterial activity of Plantago major leaves against* Streptococcus pyogenes *(ATCC 19615) as a cause of tonsilitis. Bioteknologi 17: 14-21.* Lymphatic organs are organs that play a role in the immune system. One such lymphatic organ is tonsils which are in the esophagus. Tonsils are for the body's defense against infection. Tonsils can become inflamed from bacteria or viruses. One of bacteria that causes tonsillitis is *Streptococcus pyogenes*. The prevalence of tonsillitis in various countries, especially developing countries, is high. Therefore, prevention and treatment must be carried out appropriately with the use of natural materials for treatment continues to be developed. *Plantago major* L. is a weed that has antibacterial bioactivity, so it potentially can become a tonsillitis drug. This study aims to determine the effect of variations in the concentration of *P. major* ethanol extract on the growth of *S. pyogenes* ATCC 19615 bacteria and the class of chemical compounds of *P. major* ethanol extract. The antibacterial activity test used a completely randomized design (CRD) with 7 treatment groups, namely 5 variations in the concentration of *P. major* ethanol extract, a positive control group, and a negative control group with three repetitions. Variations in concentration of *P. major* ethanol extract, namely 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL as much as 30 µl with positive control of Bacitracin disc and negative control of DMSO 10% 30 µl. The method used was the well method and the clear zone was measured using a caliper. Data analysis was carried out qualitatively and quantitatively using the Analysis of Variance One-Way Anova using SPSS. Based on the One-Way Anova test and continued with Duncan's test, a significance value of 0.000 <0.05 was obtained, thus it was said to be significant. The ethanolic extract of *P. major* was able to inhibit the growth of *S. pyogenes* ATCC 19615 at concentrations of 250 mg/mL, 500 mg/mL, and 750 mg/mL, and 1000 mg/mL. Based on the thin layer chromatography test, the ethanolic extract of *P. major* contains a class of flavonoid compounds, alkaloids, steroids, phenols, tannins, and terpenoids.

Keywords: Extract, *Plantago major*, *Streptococcus pyogenes*, tonsillitis

INTRODUCTION

Lymphatic organs are organs that play a role in the immune system, one of which is the tonsils located in the esophagus (Schedlowski and Tewes 1999). Tonsillitis is inflammation of the tonsils caused by a bacterial or viral infection. Chronic tonsillitis, which occurs frequently in children, is caused by improper antibiotic treatment (Kurien et al. 2003). Tonsillitis sufferers in Indonesia numbered roughly 23% in 2012, according to data from the Ministry of Health of the Republic of Indonesia. While ENT patients in 2012 were 3.8% for chronic tonsillitis, according to epidemiological data (Zuhdi et al. 2020).

According to WHO, Acute Respiratory Infection (ARI) is one of the diseases that can cause death, especially in children in developing countries. The prevalence of ARI in Indonesia is very high, around 21.6%, especially in urban areas. From 2000 to 2003, the number of children under five with ARI remained stable even though the ARI eradication program had been launched (Wahyuningsih et al. 2017). There is a prevalence of tonsillitis from different countries. In 1998-2007 there were 15,067 cases with a prevalence of 22% in Islamabad, Pakistan (Awan et al. 2009). The prevalence of chronic tonsillitis in the United States in 1995 was 0.7% (Novel 2010). Research on chronic tonsillitis in Russia conducted at the age of 1-15 years obtained data that as many as 84 (26.3%) were diagnosed with chronic tonsillitis and of whom had a history of tonsillectomy (Khasanov et al. 2006).

Streptococcus pyogenes is a common cause of bacterial infection in humans. When the body's defensive system weakens, these bacteria target the throat and skin, causing diseases including pharyngitis, scarlet fever, and impetigo. Invasive illnesses caused by *S. pyogenes* include muscle inflammation, bone infections, meningitis, and endocarditis (Cunningham 2000). Penicillin, beta-lactam, tetracycline, and macrolide antibiotics are all resistant against *S. pyogenes* (Pires et al. 2009).

Tonsillectomy and tonsillotomy are surgeries for treating tonsillitis that induce pain and bleeding, making them less safe, because blood volume is still low and harmful for the respiratory system at a young age (Stelter 2014). Antibiotics can be beneficial when used correctly, but if they are not used correctly (irrational prescribing), it can result in health and economic losses (Utami 2012). If the first line of antibiotics fails, the second line, third line, and so on must be used instead, which are still expensive but are more likely to result in microorganism immunity, making treatment difficult (Apua 2011). This is the basis for the need to explore natural ingredients with medicinal properties.

The broad-leaved plantain (*Plantago major* L.) is one of the medicinal plants. This plant is found in tropical and mountainous areas (Pangemanan 1999; Padua et al. 1999). The roots, stems and leaves are used as cough medicine, dysentery medicine, wound dressing, urinary stones, diabetes, gallstones, leprosy, and abdominal pain (Anon 1995). Van Stenenis-Kruseman (1963), explained that *P. major* extract was used to treat whooping cough. Research from Sharifa et al. (2008), showed that the ethanol and methanol extracts of *P. major* had antibacterial activity for both gram-negative and gram-positive bacteria. Research on the antibacterial activity of the ethanolic extract of *P. major* on *S. pyogenes* ATCC 19615 has not been carried out. Therefore, scientific research is needed to prove the antibacterial activity of *P. major*.

MATERIALS AND METHODS

Plant material

The plant material needed was *P. major*; samples collected were from plants which had flowered. This plant was obtained from Ampel Gading Hamlet, Kenteng Village, Bandungan District, Semarang Regency, Central Java. The leaves were the part of the plant that was used.

Experiment design

This study used a completely randomized design (CRD) with seven treatment groups, each with three replicates, including five different concentrations of *P. major* L ethanol extract, a positive control group, and a negative control group. Variations in the concentration of *P. major* ethanolic extract were 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL, each 30 l, with a positive control of Bacitracin antibiotic disc and a negative control of DMSO 10% 30 µl. Determination of concentration variations was based on the results of research by Razik et al. (2012).

Extraction

As much as 2.35 kg of *P. major* leaves were dried in a 50°C oven for 24 hours (Irianti et al., 2018). To make powder, the leaves were pulverized in a blender (simplicia). *P. major* leaves and stems, weighing up to 200 grams, were macerated for 5 days in 1000 cc of 96% ethanol (1:5). During the 5-day period, the solvent was changed only once. After that, it was filtered with Whatman No. 42 paper and evaporated at 50°C with a rotary evaporator (Dewi et al. 2019). At 40°C, the extract was thickened in a water bath (Irianti et al., 2018). The extract was placed in an evaporating dish and kept at 4°C in the refrigerator (Razik et al. 2012).The thick extract was weighed, and the yield was calculated using the formula:

$$\text{Yield} = \frac{\text{thick extract weight (g)}}{\text{sample weight (g)}} \text{ x } 100\ \% \text{ (Dewatisari } et\ al.\text{, 2017)}$$

Antibacterial activity test

Making Mueller Hinton blood agar media

The Nutrient Agar (NA) media was weighed to 10 grams and 2.5 grams of NaCl was added. This was then dissolved in 500 mL of distilled water, for the pH was measured to show the number 7. After that it was boiled before the solution was sterilized using an autoclave at a temperature of 121°C and a pressure of 1 atm for 15 minutes. After the media was sterile and the temperature drops to about 50°C, 20-25 mL of sheep's blood was added. Media was then poured into a petri dish (Haerazi et al. 2014).

Rejuvenation of Streptococcus pyogenes *ATCC 19615*

Mueller-Hinton Media Sterile blood agar was prepared in a petri dish and then a single loop of *S. pyogenes* ATCC 19615 was taken and streaked on the media using the streak plate method. After that, it was incubated for 24 hours at 37°C (Wirdia et al. 2017).

Antibacterial activity test of Plantago major *leaves against Streptococcus pyogenes ATCC 19615*

The antibacterial activity test was carried out in vitro with the well method and then the zone of inhibition of bacterial growth was determined. The concentration of *P. major* extract was varied, namely 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL with DMSO 60% as a solvent (Razik et al. 2012). The wells with a size of 6 mm were dripped with 30 l of extract solution. The positive control used Bacitracin discs, while the negative control used 60% DMSO. The next stage was incubation for 24 hours at 37°C. Bacterial growth was observed and the clear zone was measured using a caliper (Octaviani et al. 2019).

Phytochemical screening of ethanol extract of *Plantago major* leaves

The active compound content of the ethanolic extract of *P. major* was carried out using the Thin Layer Chromatography (TLC) method. The stationary phase was a silica gel plate G60 F254 with a length of 10 cm and a width of 2 cm. Silica gel was washed with 96% ethanol then activated in an oven at 100ºC for 60 minutes. The extract was dissolved in 96% ethanol until it was watery and then it was spotted on the stationary phase (Yuda et al. 2017).

Flavonoid test

The mobile phase used was n-hexane:ethyl acetate:methanol (4:5:1) with $AlCl_3$ as a stain. A positive reaction is indicated by the presence of a yellow to greenish-yellow stain. This indicates the presence of flavonoid compounds (Mabruroh et al. 2019; Ahmad et al. 2015).

Alkaloid test

The mobile phase used was chloroform: ethanol (24:1) with Dragendorff's stain. After being sprayed with Dragendorff, it could shows yellow or bluish spots. This

indicates the presence of alkaloid compounds (Pratita 2017).

Steroid test

The mobile phase used was chloroform: methanol (9:1) with Liebermann-Burchard reagent stains visible and then heated at 105ºC for 5 minutes. A positive reaction is indicated by the presence of a blue green stain (Kristanti et al. 2008).

Phenolic test

The mobile phase used was n-hexane:ethyl acetate (3:7) with 5% $FeCl_3$ stain. After that, it was observed with UV light at a wavelength of 254 nm and UV 366 nm. The presence of phenolic compounds would be indicated by a change in the color of the stain to blue-black or bluish-green (Fajriaty et al. 2018).

Terpenoid test

The mobile phase used was n-hexane:ethyl acetate (6:4) with a Liebermann-Burchard stain. After that, it was heated for 5 minutes at 105ºC. The presence of terpenoids would be indicated by the formation of a blue-violet or red-violet color (Hanani 2015).

Tannin test

The mobile phase used was methanol: ethyl acetate (7:3) with 5% $FeCl_3$ stain. The presence of tannin compounds would be indicated by a blue-black color and condensed tannins by the formation of a green-brown stain (Banu and Nagarajan 2014).

Observation and data collection

Observation of the antibacterial activity test was in the form of the diameter of the inhibition zone around the well after 24 hours of incubation. In the characterization of each class of chemical compounds, the color change of each spot was observed after the reagent was sprayed. In the phenol compound test, after spraying the reagent, the spot was observed with UV light at a wavelength of 254 nm and UV 366 nm.

Data analysis

The data obtained was analyzed using quantitative and qualitative analysis. Qualitative analysis consisted of data on chemical compound content of *P. major* ethanol extract and contact bioautography test, which were analyzed descriptively with data in the form of tables and figures. Quantitative analysis is data on bacterial inhibition zones. In the quantitative test, the data was analyzed using the Analysis of Variance One-Way Anova using SPSS. If there was a difference, it is continued with further testing using Duncan's test with an α=0.05 (Haerazi et al. 2014).

RESULTS AND DISCUSSION

Extraction

Plantago major samples for extraction (Figure 1) were obtained from Ampel Gading Hamlet, Kenteng Village, Bandungan District, Semarang Regency with an altitude of approximately 1300 meters above sea level (masl). The criteria for selecting *P. major* plants are those that have flowered. The parts used for extraction was all parts of the leaf. The *P. major* extraction method used the maceration method with 96% ethanol solvent. In this study, ethanol solvent was used because it is relatively safe and can attract most of the active compounds in plants so that the optimal amount of active ingredients is produced (Sulastri and Oktaviani 2015). The viscous extract obtained from the extraction process using the maceration method was 16.098 grams, while the yield in the ethanol extraction process of the leaves and stems of *P. major* was 8.049%.

Antibacterial activity test of *Plantago major* ethanol extract against *Streptococcus pyogenes* ATCC 19615

In the antibacterial activity test, 5 variations of the concentration of *P. major* ethanol extract were made, namely 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL with Bacitracin positive control and 60% DMSO negative control. The results of the inhibition are shown in Table 1 and Figure 2.

Table 1. The average diameter of the inhibition zone of the ethanolic extract of *Plantago major* against *Streptococcus pyogenes* ATCC 19615 with an incubation time of 24 hours.

Concentration (mg/mL)	Average diameter of inhibition zone (mm)
125	0
250	7,14
500	8,48
750	8,85
1000	10,57
Positive control (Bacitracin)	16,61
Negative control (DMSO 60%)	0

Figure 1. *Plantago major* L. growing in Ampel Gading Hamlet (Private collection 2020).

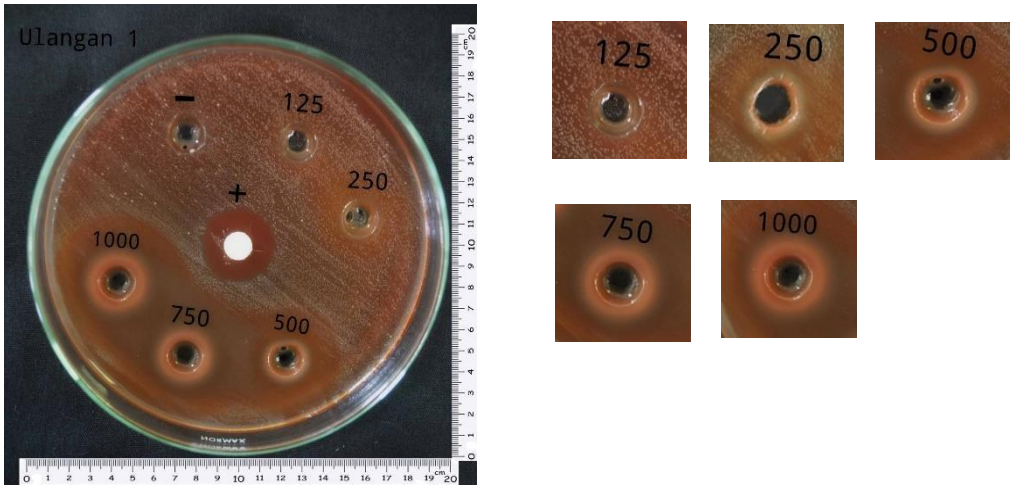

Figure 2. The results of the inhibition zone of *Plantago major* ethanol extract against *Streptococcus pyogenes* ATCC 19615 with an incubation time of 24 hours, the inhibition zone at a concentration of 125 mg/mL, the inhibition zone at a concentration of 250 mg/mL, the inhibition zone at a concentration of 500 mg/mL, the inhibition zone at a concentration of 750 mg/mL, the inhibition zone at a concentration of 1000 mg/mL.

Based on the results of One-Way Anova data analysis, there were significant differences in the diameter of the inhibition zone between the treatment groups. Data analysis continued with Duncan's test and the results showed that the negative control group (DMSO 60%) and the concentration of the ethanol extract of *P. major* 125 mg/mL were not significantly different, which indicated that the concentration had no activity to inhibit the growth of *S. pyogenes* ATCC 19615. Concentrations of 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL had inhibitory activity against the growth of *S. pyogenes* ATCC 19615, but the zone of inhibition was not as large as the positive control group (Bacitracin).

Bacitracin, according to Zaidi et al. (2020), has good antibacterial efficacy against gram-positive bacteria like *Streptococcus mutans*. The process works by preventing the formation of biofilms, which causes bacterial shape to change and cell wall synthesis to be inhibited. Bacitracin has the potential to destroy nucleic acids, hence it is frequently used for antimicrobial therapy (Ciesiolka et al. 2014).

Previous research conducted by Razik et al. (2012), showed methanol extract of *P. major* showing a zone of inhibition in gram-positive bacteria *Lactobacillus* sp. at a concentration of 125 mg/mL-1000 mg/mL and *Staphylococcus aureus* at a concentration of 250 mg/mL-1000 mg/mL. Based on the results of the study, the higher the concentration of the ethanol extract of *P. major*, the greater the zone of inhibition. Supported by the statement of Haerazi et al. (2014), that the higher the concentration of the extract, the higher the content of chemical compounds and more diffuse in the bacterial culture.

The ethanol extract of *P. major* is thought to be able to inhibit *S. pyogenes* ATCC 19615 because it contains flavonoid compounds, phenols, tannins, and terpenoids that work synergistically. Supported by research by Dewi et al. (2019), that the phenolic compounds, flavonoids, tannins, and saponins contained in the ethanol extract of *P. major* work synergistically when combined so that they are more effective at inhibiting bacteria. Sharifa et al. (2012), stated that the ethanolic extract of *P. major* caused cell shrinkage, indentation, and cell shrinkage in gram-positive (*Streptococcus aureus*) and gram-negative (*Eschericia coli*) bacteria. Supported by research by Wijesundara and Rupasinghe (2019), phytochemical compounds such as the flavonoid group, namely isoflavonoids, flavones, and phenolic compounds from licorice ethanol extract have antibacterial activity against *S. pyogenes*. Nzeako et al. (2006), also reported that clove leaf extract (*Syzygium aromaticum*) containing terpenoids, flavones, and phenolic compounds was also reported to have antibacterial activity against *S. pyogenes*.

The mechanism of inhibition of flavonoid compounds (isoflavonoids), flavones, and phenolic compounds begins with the entry of compounds through the peptidoglycan of the *S. pyogenes* bacteria; which can affect the cytoplasmic membrane resulting in structural changes in the membrane, including changes in fluidity, changes in the outer surface of the cell wall due to leakage of cytosolic fluid. This leak resulted in morphological changes to the death of *S. pyogenes* bacterial cells (Wijesundara and Rupasinghe 2019). Limsuwan et al. (2012), also mentioned that cell leakage was caused by weakening of the cell wall due to osmotic pressure during incubation.

Phytochemical screening of ethanol extract of *Plantago major* leaves

Phytochemical screening of the ethanol extract of the leaves and stems of *P. major* L. showed that the extract contained alkaloids, flavonoids, steroids, phenols, tannins, and terpenoids. According to research by Dewi et al. (2019), based on the UV-Vis Spectrophotometry test, the ethanolic extract of *P. major* L. contains phenols and flavonoids (flavones, flavanols, and aurons). While based on the analysis of phytochemical compounds, *P. major* L.

contains phenols, flavonoids, saponins, and tannins. According to research Adom et al. (2017), groups of chemical compounds in *P. major* L. are flavonoids, alkaloids, terpenoids, phenols, iridoid glycosides, fatty acids, polysaccharides, and vitamins.

Alkaloid test

A group of alkaloid compounds contained in the ethanol extract of *P. major* was detected with Dragendorff's reagent. The results of spraying reagents can be seen in Figure 3.

The mobile phase used was chloroform: ethanol (24:1). After spraying Dragendorff's reagent, spot C (Rf 0.71) changed color from yellow to bluish which indicates that the ethanol extract of *P. major* contains alkaloids. Based on the results of the elution, there were four spots (spots), namely spot A (Rf 0.23), spot B (Rf 0.34), spot C (Rf 0.71), and spot D (Rf 1.03).

The color change in the spot occurs because the alkaloid compound has a nitrogen group and there is one pair of free electrons, so that this compound can bind positively charged metal ions and then form complex compounds. Dragendorff's reagent for the alkaloid test was made using nitrogen to form a coordinate covalent bond with K+ which is a metal ion so that the stain color changes to yellow or bluish (Figure 4) (Marliana et al, 2005). The group of alkaloid compounds has biological activities, namely antimalarial, anticancer, antimicrobial, antioxidant, and anti-inflammatory (Ernawati et al. 2018).

Since the alkaloid compound has a nitrogen group and one pair of free electrons, the spot changes color. This allows the molecule to attach positively charged metal ions and build complex compounds. Dragendorff's reagent for the alkaloid test uses nitrogen to establish a coordinating covalent bond with K^+, a metal ion, causing the stain to turn yellow or bluish in color (Figure 4) (Marliana et al. 2005). Antimalarial, anticancer, antibacterial, antioxidant, and anti-inflammatory properties are among the biological activities of alkaloid substances (Ernawati et al. 2018).

Flavonoid test

The group of flavonoid compounds contained in the ethanol extract of *P. major* was detected with $AlCl_3$ reagent. The results of spraying reagents can be seen in Figure 5.

The mixture of n-hexane:ethyl acetate:methanol was utilized as the mobile phase (4:5:1). The color of spot E (Rf 1.01) changed to yellow after spraying with $AlCl_3$ reagent, showing that the ethanol extract of *P. major* contains a family of flavonoid chemicals. There were six areas identified based on the elution results: spot A (Rf 0.25), spot B (Rf 0.5), spot C (Rf 0.6), spot D (Rf 0.66), spot E (Rf 1, 01), and spot F (Rf 1, 01). (Rf 1.05).

The color change in the spot occurs due to a chemical reaction with AlCl3 reagent. This reagent will react with flavonoids to form complex compounds. The AlCl3 reagent detects the presence of an o-hydroxy group on the C-4 atom and an o-hydroxy ketone group on the C-3 or C-5 atom (Figure 6), therefore there will be a color change in the stain to yellow (Azizah et al. 2014). According to Saravanakumar et al. (2009), flavonoids have pharmacological, antimicrobial, antioxidant, cytotoxic, and antiproliferative activities.

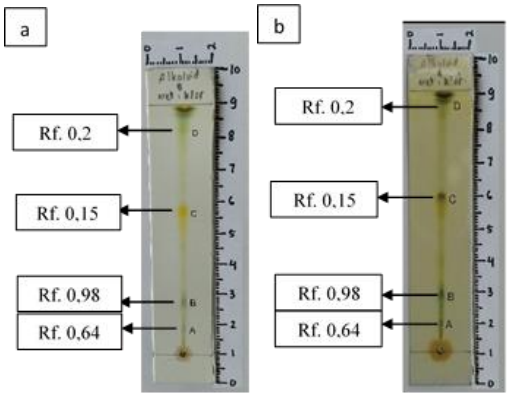

Figure 3. Chromatogram of *Plantago major* ethanol extract with silica gel plate G60 F254 as stationary phase and chloroform: ethanol (24:1) mobile phase, a. Before spraying Dragendorff's reagent, b. After spraying Dragendorff's reagent.

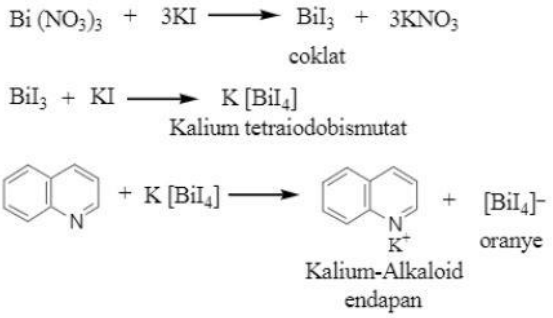

Figure 4. Dragendorff reagent reaction (Marliana et al. 2005).

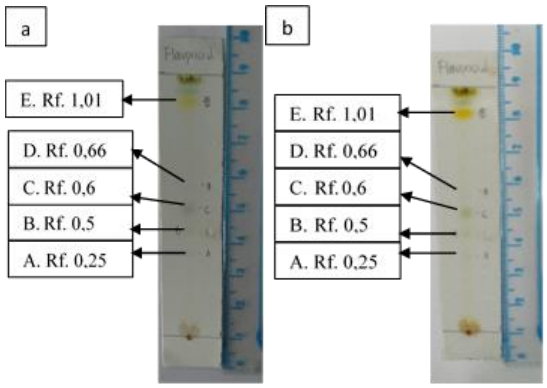

Figure 5. Chromatogram of *Plantago major* ethanol extract with silica gel plate G60 F254 as stationary phase and n-hexane:ethyl acetate:methanol (4:5:1) mobile phase, a. Before spraying $AlCl_3$ reagent, b.After spraying $AlCl_3$ reagent.

Steroid test

The group of steroid compounds contained in the ethanol extract of *P. major* was detected with Liebermann-Burchard reagent, accompanied by heating at a temperature of 105ºC for 5 minutes. The results of spraying reagents can be seen in Figure 7.

The mobile phase used was chloroform: methanol (9:1). The color change in the spot occurs due to the extension of the conjugation caused by the release of the hydrogen group when the steroid compound reacts with the Liebermann-Burchard reagent (Siadi 2012). Based on the results of the elution, there were six spots (spots) namely spot A (Rf 0.16), spot B (Rf 0.46), spot C (Rf 0.71), spot D (Rf 0.8), E (Rf 0.86), and F (Rf 0.92). The steroid compound group has antibacterial, antifungal, and antidiabetic activity (Hidayah et al. 2016).

Phenolic test

The group of phenolic compounds contained in the ethanol extract of *P. major* was detected with 5% $FeCl_3$ reagent. The results of spraying reagents can be seen in Figure 8.

The mobile phase used was n-hexane:ethyl acetate (3:7). After being sprayed with 5% FeCl3 reagent, there was a color change in spots B (Rf 0.35) and C (Rf 1.01) from yellow to blackish blue, indicating that the ethanol extract of *P. major* contains a group of phenolic compounds. The color change occurs due to the formation of complex compounds between phenol and Fe, which are metal elements (Wardhani and Sulistyani 2012).

Based on the results of the elution, there were four spots (spots), namely spot A (Rf 0.35), spot B (Rf 0.5), spot C (Rf 1.01) and spot D (Rf 1.06). After being observed with UV light at a wavelength of 254 nm, the spot shows a blue-black color while at a wavelength of 366 nm, the spot is not visible. According to Gandjar et al. (2007), stains will appear at wavelengths of 254 nm and 366 nm because these wavelengths interact with the fluorescence indicator of the TLC plate. This plate component has light emission that is emitted when electrons move from low energy to higher energy then return to their original state by releasing energy.

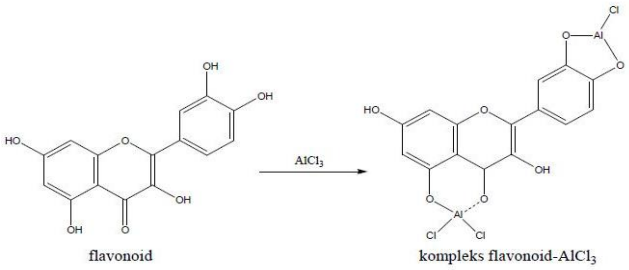

Figure 6. Reaction for the formation of complex compounds between flavonoids and $AlCl_3$ (Dewi et al. 2018).

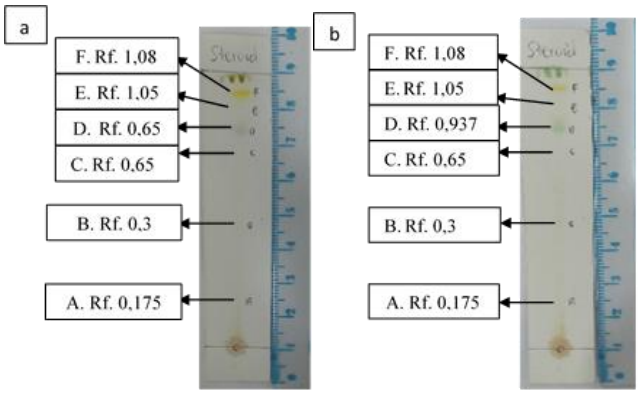

Figure 7. Chromatogram of *Plantago major* ethanolic extract with silica gel plate G60 F254 as stationary phase and chloroform: ethanol (24:1) mobile phase, a. Before spraying Liebermann-Burchard reagent accompanied by heating at a temperature of 105°C for 5 minutes, b. After spraying Liebermann-Burchard reagent then heated at a temperature of 105°C for 5 minutes.

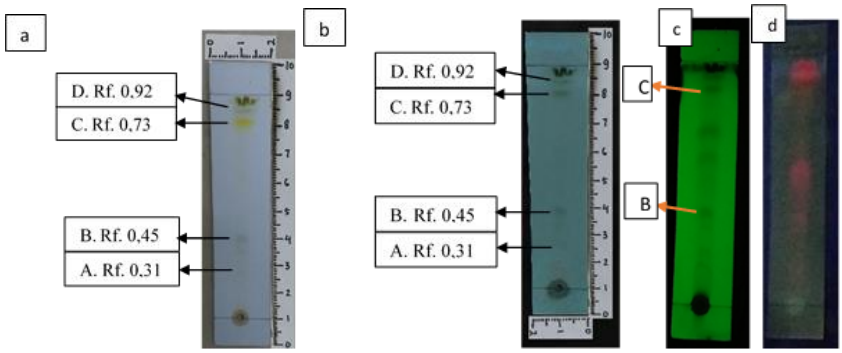

Figure 8. Chromatogram of *Plantago major* ethanol extract with a stationary phase of silica gel plate G60 F254 and a mobile phase of n-hexane: ethyl acetate (3:7), a. Before spraying 5% $FeCl_3$ reagent b. After spraying 5% $FeCl_3$ reagent, c. Fluorescence results at a wavelength of 254 nm after being sprayed with 5% $FeCl_3$ reagent, d. Fluorescence results at a wavelength of 366 nm after being sprayed with 5% $FeCl_3$ reagent.

A spot that glows under UV light at 254 nm shows the presence of two conjugated double bonds in the molecule, according to Alen et al. (2017). Spots that glow under 366 nm UV light have a longer conjugated double bond or chromophore (an organic molecule with a conjugated double bond that absorbs color) and an auxochrome group. Phenolic chemicals, according to Carolia and Noventi (2016), act as antibacterials by denaturing proteins and bacterial cells.

Tannin test

The group of tannin compounds contained in the ethanol extract of *P. major* was detected with 5% $FeCl_3$ reagent. The results of spraying reagents can be seen in Figure 9. The mobile phase used was methanol: ethyl acetate (7:3). After being sprayed with 5% FeCl3 reagent, there was a color change in spot B (Rf 0.81) and C (Rf 0.96) from yellow to blackish, indicating that the ethanol extract of *P. major* contains a class of tannin compounds. Based on the results of the elution, there are three spots (spots): spot A (Rf 0.71), spot B (Rf 0.81), and spot C (Rf 0.96).

The color change in the spot occurs because the $FeCl_3$ reagent reacts with the hydroxyl group on the tannin compound, resulting in hydrolysis of the tannin group and produces a blue-black color change, while the condensed tannin changes color to blackish green (Pardede et al. 2013). According to Sa'adah (2010), tannin and $FeCl_3$ compounds form complex compounds with Fe^{3+} ions. According to Ergina et al. (2014), if the test results for phenol compounds are positive, it is possible that one of the compounds is tannin. This is because tannins are polyphenolic compounds. Maharani et al. (2017) mentioned that tannins have antibacterial, antioxidant, and anti-diarrheal activities.

Terpenoid test

The terpenoid compounds contained in the ethanol extract of *P. major* were detected with Liebermann-Burchard reagent and then heated at 105 ºC for 5 minutes. The results of spraying reagents can be seen in Figure 10.

The mobile phase used was n-hexane:ethyl acetate (6:4). After being sprayed with Liebermann-Burchard reagent and then heated at 105 ºC for 5 minutes, there was a color change in spot E (Rf 0.94), spot F (Rf 1), and spot G (Rf 1.11) from yellow to red-violet. which showed that the ethanol extract of *P. major* contained a class of terpenoid compounds. The color change in the spot occurs due to the extension of the conjugation caused by the release of the hydrogen group when the terpenoid compound reacts with the Liebermann-Burchard reagent (Siadi 2012).

Based on the results of the elution, there were seven spots (spots), namely spot A (Rf 0.54), spot B (Rf 0.65), spot C (Rf 0.7), spot D (Rf 0.84), spot E (Rf 0.94), spot F (Rf 1), and spot G (Rf. 1.11). According to Wu et al. (2020), terpenoids have biological activities such as anti-inflammatory, antimicrobial, anticancer, antioxidant, and immunomodulatory. Irianti et al. (2018), stated that the class of terpenoid compounds affects the permeability of bacterial cell membranes.

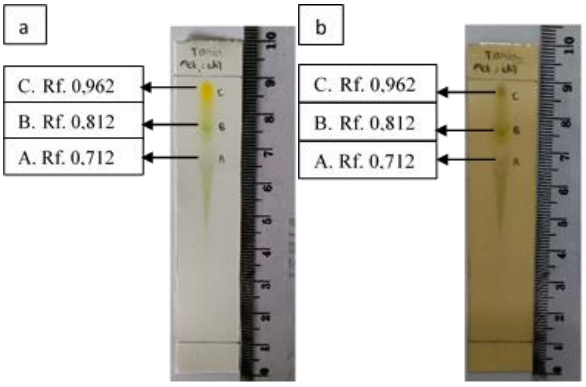

Figure 9. Chromatogram of *Plantago major* ethanol extract with silica gel plate G60 F254 as stationary phase and methanol as mobile phase: ethyl acetate (7:3), a. Before spraying 5% $FeCl_3$ reagent b. After spraying 5% $FeCl_3$ reagent.

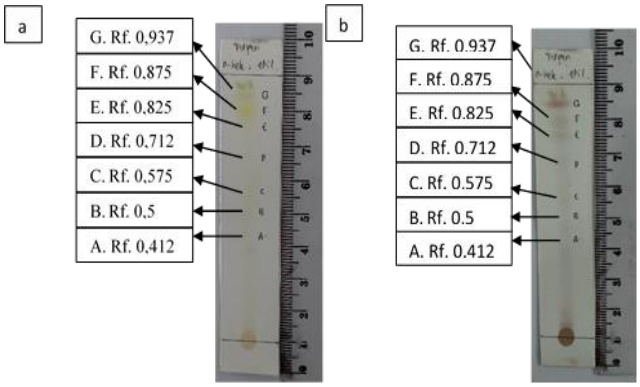

Figure 10. Chromatogram of *Plantago major* ethanol extract with G60 F 254 stationary phase and n-hexane as mobile phase: ethyl acetate (6:4), a. Before spraying Liebermann-Burchard reagent then heated at 105oC for 5 minutes, b.After spraying Liebermann-Burchard reagent then heated at 105ºC for 5 minutes.

The conclusion of this study is that the ethanolic extract of *P. major* at concentrations of 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL had an inhibitory effect on the growth of *S. pyogenes* ATCC, while at concentrations of 125 mg/mL and DMSO 60% negative control had no effect. Groups of chemical compounds in the ethanol extract of *P. major* based on thin layer chromatography tests are flavonoids, alkaloids, terpenoids, tannins, steroids, and phenols.

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