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Antibacterial Activity of Seligi Leaf Extracts and Fractions against Pseudomonas Aeruginosa and Staphylococcus Aureus Bacteria and Their Bioautography

Rizky Wanda Prastika[1], Cita Hanif Muflihah[1]

[1]*Fakultas Farmasi, Universitas Muhammadiyah Surakarta*

**Corresponding Author: Rizky Wanda Prastika*
E-mail: chm641@ums.ac.id

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Abstract

Seligi leaf (Phyllanthus buxifolius muell. Arg) is one of the medicinal plants that are traditionally used by the community. This plant can empirically treat several diseases, thanks to the content of antibacterial compounds such as flavonoids and terpenoids. The bacteria Pseudomonas aeruginosa and Staphylococcus aureus are the main causes of nosocomial infections. The purpose of this study is to examine the antibacterial activity of seligi leaf extract and fraction against Pseudomonas aeruginosa and Staphylococcus aureus bacteria, as well as to identify active compounds that have an important role in these antibacterial activities. The extraction process is carried out using the maceration method with 70% ethanol. The antibacterial activity test was carried out by the disc diffusion method, with concentration of 20, 40, 60, 80, and 100% for extraction, then for all fractions a concentration of 50 %. The test results showed that the extract had antibacterial activity against Staphylococcus aureus on 8 mg/disk loading disk with inhibition zone diameters of 10.33 ± 0.58 mm and 12.16 ± 1.41 mm, but no inhibition zones formed against Pseudomonas aeruginosa on all loading disks. The identification of compounds in seligi leaves using the KLT method showed that the extract contained phenolics, flavonoids, and terpenoids, while the ethyl acetate fraction contained phenolics and alkaloids. However, bioautographic tests do not indicate any antibacterial potential.

Introduction

Infectious diseases are diseases caused by bacteria, viruses, and parasites. In Southeast Asia, the most common infection is nosocomial infection, which usually appears in the hospital within 72 hours of the patient being treated. The World Health Organization (WHO) reported in 2009 that 8.7% of nosocomial infections occurred in 55 hospitals spread across 14 countries, including Southeast Asia, Asia Pacific, Europe and the Middle East (Baharutan et al., 2015). Pseudomonas aeruginosa is a type of bacteria including Gram-negative which is an opportunistic pathogen. These bacteria can result in nosocomial infections in patients, the use of catheters causes urinary tract infections and pneumonia. Staphylococcus aureus is a Gram-positive bacterium that can be harmful to humans when the immune system is weakened due to hormonal changes, and steroid use, in addition to Staphylococcus aureus is also known as the main cause of infections worldwide, it can result in various levels of infections ranging from infections of the skin, urinary tract, and respiratory tract to infections of the eyes and central nervous system (Afifurrahman et al., 2014). Antibiotics are antimicrobial drugs used for the treatment of bacterial infections. The incidence of various infectious diseases in Indonesia is quite high, with an increase in bacterial resistance to antibiotics (Gunawan &

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Tjandra, 2021). As an effort to overcome this, an alternative search was carried out to use herbal medicinal plants that contain antibacterial compounds.

Seligi (Phyllanthus buxifolius muell. Arg) as a type of plant in the species Phyllanthus can be found in Indonesia and is known as a traditional medicinal plant that is rich in benefits. One of its uses is as an antibacterial agent that has a class of active compounds such as alkaloids, flavonoids, tannins, saponins, quinones, steroids and triterpenoids (Wardah et al., 2007). A study shows that Phyllanthus buxifolius muell. Arg has many pharmacological activities such as antibacterial, antioxidant, antiviral, anti-inflammatory, antiallergic and anticancer (Gusnedi, 2013). Results of phytochemical tests on the findings of Sunarmi & Suhendriyo (2023) seligi leaf extract (Phyllanthus buxifolius muell. Arg) showed positive results containing tannins, alkaloids but not flavonoids.

Research by Diah Kemala Sari & Siwi Hastuti (2010) proved that the determination of total flavonoid levels of ethanol extract of seligi leaves was determined using a quercetin comparator in a concentration series of 50, 100, 200, 300, 400 ppm with a wavelength of 480 nm, the absorbance value measured using a UV-Vis spectrophotometer. The results of the study obtained flavonoid levels of ethanol extract of seligi leaves were (13.86 ± 1.27%). The difference between the previous study and the research to be carried out is that the materials used are seligi leaf extract with 70% ethanol using the UV-Vis spectrophotometry method, while for this study it uses seligi leaf extract with 70% ethanol and the maceration method as an antibacterial activity by the disk diffusion method and the determination of compound groups using the KLT method.

Based on the previous description, no studies have been found that test the antibacterial activity of ethyl acetate extracts and fractions, n-hexane, and methanol of seligi leaves (Phyllanthus buxifolius muell. Arg) against Pseudomonas aeruginosa and Staphylococcus aureus bacteria by bioautographic test. Therefore, the research conducted can be held with the aim of determining the antibacterial activity of extracts, seligi leaf fractions in Pseudomonas aeruginosa and Staphylococcus aureus bacteria, as well as identifying the active compounds they contain using KLT and conducting bioautography.

Methods

Tool

In this study, the necessary tools are an oven (Memmert), a refrigerator, a blender, an analytical balance (Ohaus), a maceration vessel, *a rotary evaporator* (Stuart), a waterbath (Mammert), a separate funnel, an autoclave (Hirayama), *a laminar air flow* (LAF) (Astari niagara), *a shaker incubator* (Excella E24 Incubator Shaker Series), an incubator (Memmert), a glass beaker, a bunsen, a measuring cup, a tube rack, tweezers, *a glass* spreader, Erlenmeyer, magnetic stirrer, hot plate, petri cup, test tube, round ose, 1000 µL micropipette (Socorex), 10 µL micropipette (Socorex), ruler, 10 cc syringe, UV lamp 254 nm and 366 nm .

Material

There are several materials needed in the study carried out including seligi leaves obtained in the Tawamangu area, *Pseudomonas aeruginosa bacteria* from the microbiology laboratory of the Faculty of Pharmacy UMS, *Staphylococcus aureus* bacteria from the same laboratory, and ethanol 70% pro-analysis (p.a), ethyl acetate (p.a), n-hexane (p.a), formic acid (p.a), *aquadest*, sodium chloride (NaCl) 0.9% sterile, *dimethyl sulfoxide* (DMSO), 0.5 *McFarland standard* (1.5 x 10^8 CFU/mL), *Brain Heart Infusion* (BHI) (Oxoid), *Mueller Hinton agar* (MHA) (Oxoid), Whatman filter paper no 41, *yellow tips*, *blue tips*, *white tips*, *blank disc* (Liofilchem),

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silica gel plate GF254, chloramphenicol 30 μg antibiotic (Oxoid), ampicillin 10 μg (Oxoid), erythromycin 15 μg (Oxoid), vancomisin 30 μg (Oxoid), as well as Sitroborate, Dragendorff, Liebermann-Burchard, and FeCl3 spray reagents.

Research procedure

Determined

(Klau & Hesturini 2021). *Phyllanthus buxifolius Muell. Arg*) yang dipergunakan pada studi ini telah dideterminasi secara resmi oleh Balai Besar Penelitian, Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT) Tawangmangu, Karanganyar. (Klau and Hesturini 2021).

Extraction

Seligi leaf powder weighing 500 grams is placed in a dark container and dissolved using 70% ethanol (p.a) as much as 5000 ml. The mixture is placed at room temperature protected from sun exposure for 5 days and stirred once a day. After that, the solution is filtered through Whatman No. 41 filter paper. The simplicia of seligi leaves was then remachelized once with a similar method and the results were combined. The filter results from the reclamation are evaporated at a temperature of 40℃ to separate the solvent from the extract, and then concentrated with *a water bath* at a temperature of 50℃ until the extract thickens (Agustningsih et al., 2014). The thickened extract is weighed to calculate its yield, then stored in the refrigerator.

Fraksinasi

A thick extract from the leaves of seligi (*Phyllanthus buxifolius.muell.Arg*) of 2 grams is weighed, then dissolved in 30 ml of methanol:water solvent mixture in a ratio (1:1). The mixture is poured into a separate funnel, followed by the addition of 30 ml of non-polar (n-hexane) solvent, then shaken and let stand until it forms the top (n-hexane) and bottom (methanol:water) layers. This process is repeated up to 3 times with a total of 6 grams of extract and 90 ml of non-polar (n-hexane) solvent. The separated layer is transferred to a different container, then the solvent layer (methanol:water) is put again in the separation funnel. Next, 30 ml of semi-polar solvent (ethyl acetate) is added, shaken, and let stand until the upper (ethyl acetate) and bottom (methanol:water) layers are formed. This process is repeated 3 times with a total of 90 ml of semi-polar solvent (ethyl acetate). The three solvent fractions are then dried using a water bath at a temperature of 50℃ until the extract thickens, then the yield is calculated and stored in the refrigerator (Hatur Rahman, 2021).

Sterilization of Tools and Materials

The glassware used for antibacterial research, namely test tubes and petri dishes, is first sterilized with an oven at a temperature of 180℃ within 2 hours. Meanwhile, micropipette tips and sterilization media with autoclave at 121℃ within 1 hour, while tools such as tweezers, *glass spreaders* and round ose are sterilized using bunsen fire burning (Paputungan et al., 2019).

Media Creation

MHA (*Mueller Hinton Agar*) media weighing 3.8 grams is dissolved into 100 ml of aquades, while BHI (*Brain Heart Infusion*) weighing 5.2 grams is dissolved in 100 ml of aquades. The two solutions are heated on a *hot plate* and stirred using *a magnetic stirrer* until homogeneous. Then, the sterilization solution is carried out by *autoclave* within 15 minutes at a temperature of 121℃. The medium is then poured into a sterile petri dish, and the BHI is poured into a test

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tube. The waiting process is carried out until the media hardens, with the pouring of the media carried out in the *Laminar Air Flow* (LAF) (Wardoyo et al., 2020).

Pure Bacterial Culture Rejuvenation and Bacterial Suspension Manufacturing

To rejuvenate bacterial cultures, bacteria were taken from one colony of test bacteria and scratched to form a zig-zag on MHA media for *Staphylococcus aureus* and other MHA media for *Pseudomonas aeruginosa* using the streak plate method. Furthermore, the creation of a bacterial suspension is carried out by moving 3-5 bacterial colonies into a sterile test tube and then suspending them into a test tube containing 5ml of BHI liquid. This suspension is then incubated in a *shaker incubator* for 18-24 hours until it becomes cloudy. After incubation, 200 *µL was taken and 0.9% sterile NaCl was added, then harmonized to the standard of 0.5 McFarland* (1.5 x 108 *CFU/mL*) (Undap et al., 2019).

Bacterial Sensitivity Test

The planting medium using MHA was poured 200 µl of suspension using a micropipette and added 0.9% NaCl poured in the medium and scraped thoroughly with a *glass spreader*. Antibiotic disks such as ampicillin, vancomisin, erythromycin, and chloramphenicol are placed on the surface of the media that has been implanted with bacteria, then incubated within 24 hours. The ability of antibiotics is evidenced by the formation of a clear zone around the disc attached to the medium (Agustina et al., 2019).

Antibacterial Activity Test

The antibacterial activity test method through *a diffusion disk* with MHA medium of bacteria was poured 200 µl of suspension using a micropipette and added 0.9% sterile NaCl and thoroughly flattened with a *spreader glass*, then a sample solution of seligi leaf extract with a concentration of 20, 40, 60, 80, 100 % b/v was made into DMSO with the addition of 10 µL to each disk so that *the loading dose* The doses used were 2 mg/disk, 4 mg/disk, 6 mg/disk, 8 mg/disk and 10 mg/disk. In the ethyl acetate fraction, the n-hexane fraction and the methanol fraction were made at a concentration of 50%, the negative control used ethanol was 70%, the positive control used antibiotics which produced the largest diameter in the sensitivity test. A 100% extract solution is made by considering 5 grams of thick extract and then dissolving it in 5 mL of DMSO. The sample solution is dripped on a blank disk (*liofilchem*) and left for 30 minutes so that the sample solution is fully absorbed, then the blank disk is pasted in a medium that has been inoculated with test bacteria and the medium is incubated at a temperature of 37ºC within 24 hours (Qolbi & Yuliani, 2018).

Uji Chromemography Lapis Tipis (KLT)

The GF254 silica gel KLT plate was cut into 1x7cm sizes and given a mark of the upper and lower edges with a distance of 1cm as a marker of the initial position of the point and the final boundary of the elution. The plates are then activated by heating them in the oven for 30 minutes at 100ºC. Then, each extract and fraction *of Phyllanthus buxifolius* is applied with a 1 µl micropipette at the starting point of the point, and placed into a chamber containing the saturated phase of motion. The motion phase used to dispense the extract is n-hexane: ethyl acetate (6:4 v/v), while for the ethyl acetate fraction, the motion phase used is ethyl acetate: methanol: formic acid with a ratio (6:4:1). After reaching the final limit of the elution, the plate is removed and observed with UV lamps whose wavelengths are 366 nm and 254 nm. Identify spots that form using various spray reagents, for example citroborate for flavonoids, Dragendorff for alkaloids, Liebermann-Burchard for terpenoids, and FeCl3 for phenolic compounds (Sunarmi, & Suhendriyo, 2023).

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The Bioethic

KLT plates with GF254 silica gel were cut into 1x7cm sizes and marked with the upper and lower edges at a distance of 1cm as a sign of the initial position and the final limit of the elution process. Then the plates are activated by heating them in the oven within 30 minutes at a temperature of 100ºC. Each extract and fraction of the seligi leaf (*Phyllanthus buxifolius muell. Arg)* is dosed using a 2 µl micropipette at the starting point of the tote, and placed in a chamber containing the motion phase that has been saturated. After reaching the end limit of the elucidating process, the plates are removed and then observed with UV lamps with wavelengths of 366 nm and 254 nm. As a negative control, KLT plates were also diluted without being given extract or fraction spikes. The KLT plate is then glued over the medium (the part of the silica touching the agar) that has been inoculated with test bacteria for 30 minutes until the KLT plate adheres perfectly. After that, the KLT plates are taken and incubated at 37°C within 24 hours. The results were observed by taking into account *the Retardation Factor* (Rf) of the inhibitory zone formed in the KLT plate, and the Rf was compared with the Rf of the compound group content in the identification with the KLT (Paputungan et al., 2019).

Data Analysis

Analysis of extract results and fractions of seligi leaves (*Phylanthus buxifolius muell. Arg*) calculated using the formula (Sari & Hastuti, 2020) :

$$\text{Rendeming} = \frac{The\ weight\ obtained\ by\ the\ extract}{simple\ weight} \text{ x } 100\%$$

The results of the antibacterial activity test of the extract and fraction of seligi leaves (Phyllanthus buxifolius Muell.Arg) against Pseudomonas aeruginosa and Staphylococcus aureus bacteria are evidenced by the presence of a clear zone formed around the disk (Paputungan et al., 2019). Analysis using TLC-bioautography involves comparing the Retardation Factor (Rf) value in the inhibition zone with the Rf spot on the TLC plate during phytochemical testing. Rf is explained as the ratio between the distance traveled by the compound in the stationary phase divided by the distance traveled by the solvent in the mobile phase (Mulqie & Anggadireja, 2020). The Rf calculation formula is:

$$\text{Rf} = \frac{distance\ of\ the\ center\ of\ the\ spot\ from\ the\ spot}{development\ distance}$$

Results and Discussion

Determination Results

The determination is carried out with the aim of ensuring whether the sample to be used is valid in the research. Plant determination is carried out by adjusting the morphological characteristics contained in the seligi plant *(Phyllanthus buxifolius muell. Arg)* the plant parts taken during the determination test, namely roots, stems, leaves, and fruits, the results of the determination stated that the plant carried out by the study was the species *Phyllanthus buxifolius (Blume) mull. Arg* belonging to the *phyllanthaceae* family (Ekayani et al., 2021).

Extraction and Fractionation Results

The purpose of extraction is to isolate a substance from other mixtures. In this process, a 70% ethanol solvent (p.a) is used. As a result, a thick extract weighing 86.60 grams was obtained from 500 grams of lily leaf powder, resulting in a yield of 17.92% (Mangunwardoyo et al., 2009). Fractionation is a technique for separating and classifying the chemical content in an extract based on its polarity level. In the liquid-liquid fractionation process, the concentrated extract is fractionated with three solvents at different levels of polarity: n-hexane, ethyl acetate,

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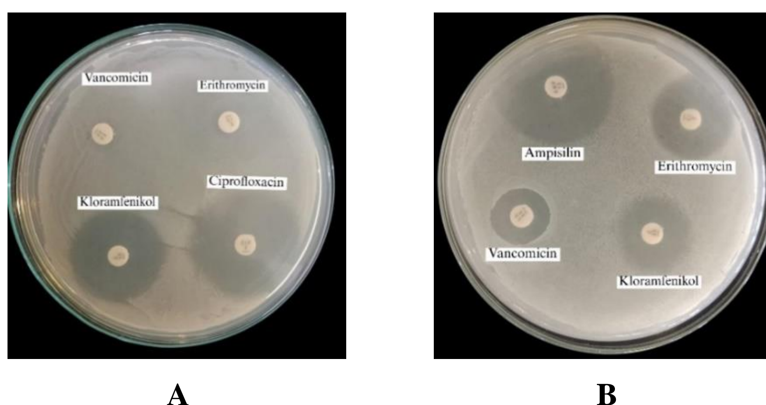
and methanol. When placed in a separate funnel, the solvent forms two different phases the upper layer consists of a solvent with a lower density and the lower layer consists of a solvent with a higher density (Paputungan et al., 2019). The three fractional solutions that were separated were dried using *a water bath* to thicken the extract, but for the *Phyllanthus buxifolius plant* with the three solvents did not produce a thickened extract because the possibility of evaporation was not maximized because the evaporation time was not long so that it could affect the physical properties, including for the thickening process, so there was no yield in the fraction. The volume of the liquid fraction obtained was 1 ml of methanol, the volume of the liquid fraction of ethyl acetate was 1 ml, and the volume of the liquid fraction of n-hexane was 1 ml. The largest fraction result in the antibacterial activity test was the ethyl acetate fraction. The ethyl acetate fraction is a semi-polar solvent that has the ability to attract a class of compounds with a wide polarity range from polar to nonpolar.

Sensitivity Test Results

Sensitivity test is a method that measures the level of sensitivity of bacteria to the antibiotics used. In this study, the sensitivity test on *Staphylococcus aureus bacteria* was carried out using an antibiotic disc of ampicillin 10 µg, chloramphinol 30 µg, vancomisin 30 µg, and erythritomycin 15 µg. The sensitivity test on *Pseudomonas aeruginosa bacteria* used an antibiotic disc of ciprofloxacin 5 µg, chloramphenicol 30 µg, vancomisin 30 µg, and erythromycin 15 µg. The results of the sensitivity test were observed through the diameter of the inhibition zone with a sensitivity standard to antibiotic.

Table 1. Sensitivity test results for *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria

Bacteria	Antibiotics	Standard sensitivity (mm)			Result	
		Sensitive	Intermediate	Resisten	Inhibition zone	Information
P. aeruginosa	Ciprofloxacin 15 µg	≥ 21	16-20	≤ 15	34,5	Sensitive
	Kloramfenikol 30µg	≥ 18	13-17	≤ 12	27	Sensitive
	Vancomicin 30 µg	-	-	-	6	
	Erithromycin 15 µg	≥ 18	14-17	≤ 13	9	Sensitive
S. aureus	Ampisilin 10 µg	≥ 21	16-20	≤ 15	32,7	Sensitive
	Kloramfenikol 30 µg	≥ 18	13-17	≤ 12	21,5	Sensitive
	Vancomicin 30 µg	-	-	-	15	
	Erithromycin 15 µg	≥ 18	14-17	≤ 13	23,3	Resist



A **B**

Figure 1. Results of antibiotic sensitivity, (A) Pseudomonas aeruginosa bacteria, (B) Staphylococcus aureus bacteria

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Based on the data recorded in Table 2, the bacterium *Pseudomonas aeruginosa* produced the largest inhibitory zone diameter in the ciprofloxacin antibiotic 5μg, reaching 34.55 mm. *Staphylococcus aureus* bacteria produce the largest inhibitory zone at ampicillin 32.7 mm, but the potential of antibacterial antibiotics can be said to be effective if the diameter of the resulting inhibitory zone is in the sensitive category. Therefore, for the results of the sensitivity test on *Staphylococcus aureus* bacteria, chloramphenicol antibiotics were used 30 μg. The mechanism of action of chloramphenicol is that it can block the activity of the peptidyl transferase enzyme in the elongation phase which causes disturbances in bacterial protein synthesis. Based on the results of the sensitivity test, the antibiotic ciprofloxacin 5μg showed the largest inhibitory zone, so this antibiotic was chosen as a positive control in the study. The antibiotic ciprofloxacin works by inhibiting the DNA enzymes gyrase and topoisomerase, so ciprofloxacin is often effective against the bacterium *Pseudomonas aeruginosa* (Shariati et al., 2022).

Antibacterial Activity Test Results

In this study, the method used to test the antibacterial activity of seligi leaf extract and fraction (*Phyllanthus Buxifolius Muell. Arg*) is a disc diffusion using *Mueller Hinton Agar* (MHA) media that has been inoculated with a testing bacterium. In this experiment, the disk containing the test sample is placed with varying concentrators. The antibacterial activity test of seligi leaf extract used concentrations of 40, 60, 80, and 100% with negative DMSO control, DMSO solvent was chosen because it can dissolve various types of compounds, both polar and non-polar, and does not have an inhibition effect on bacterial growth so that it does not affect the observation results in the antibacterial activity test (Handayani et al., 2009). Activity tests on seligi leaf fractions include ethyl acetate fraction, methanol fraction and n-hexane fraction with a negative control of 70% ethanol, 70% ethanol solvent was chosen as a negative control because the fraction results could not thicken so the negative control used was by adjusting the solvent from the extraction. Positive control in *Pseudomonas aeruginosa* bacteria with a 5 μg ciprofloxacin antibiotic disc obtained in the sensitivity test by looking at the sensitivity standard in antibiotics, while in *Staphylococcus aureus* bacteria used a 30 μg chloramphenicol antibiotic disc obtained in the sensitivity test by looking at the sensitivity standard in antibiotics. Chloramphenicol works by inhibiting the enzyme peptidyl transferase, which plays a role in the formation of peptide bonds between new amino acids that are still bound to tRNA and the last amino acid that is being synthesized (Pratiwi, 2008). Based on this information, the antibiotic chloramphenicol 30 μg was selected as a positive control in the antibacterial activity test.

The antibacterial activity test is assessed by observing the creation of clear zones or inhibition zones around the paper discs in the growth medium. The diameter of the inhibition zone is measured with a ruler, and the average diameter is calculated. The results of testing the antibacterial activity of seligi leaf extract and fraction (*Phyllanthus buxifolius Muell.Arg*) on *Pseudomonas aeruginosa* and *Staphylococcus aureus bacteria* are presented in Table 3, Figure 2, and Figure 3.

Table 1. Results of the antibacterial activity test

Bacteria	Inhibition zone diameter (mean ± SD mm)										
	Extract concentration (mg/disc)						Fraction concentration (mg/disk))				
	4	6	8	10	K+	K-	M	EA	H	K+	KF-
P Aeruginosa	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	28,67 ± 0,58	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	22,67 ± 0,58	6,0 ± 0,0

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S Aureus	8,66 ± 0,58	10 ± 0,87	10,33 ± 0,58	12,16 ± 1,41	20 ± 1	6,0 ± 0,0	6,0 ± 0,0	8,33 ± 0,58	6,0 ± 0,0	21,33± 1,15	6,0 ± 0,0

Description: Diameter including disk size 6 mm

K+ = Ciprofloxacin (Pseudomonas aeruginosa), Chloramphenicol (Staphylococcus aureus)

K- = DMSO

EA = Ethyl acetate fraction

M = Methanol fraction

H = N-hexane fraction

KF- = Ethanol 70%

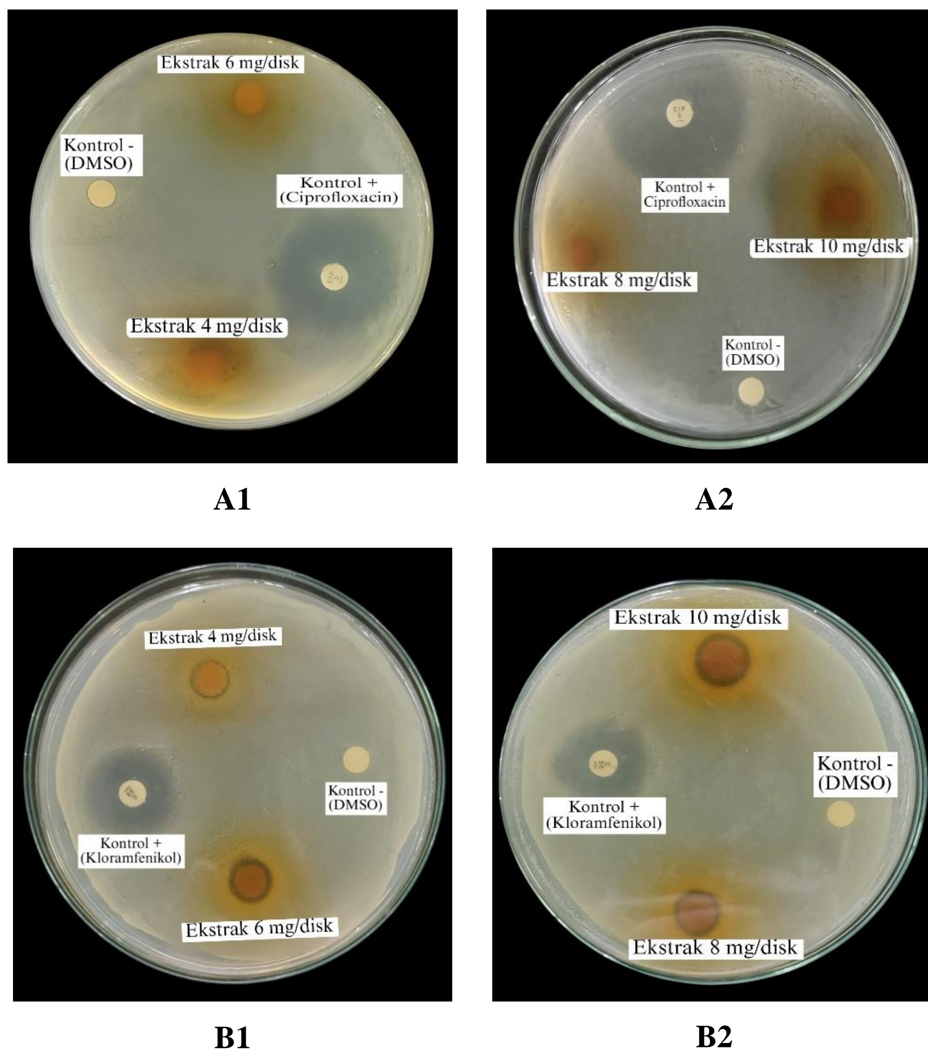

Figure 2. Results of the activity test of seligi leaf extract (Phyllanthus buxifolius muel. Arg) in 70% ethanol solvent, (A1) extract concentration of 4 mg/disk and 6 mg/disk, (A2) extract concentration of 8 mg/disk and 10 mg/disk against Pseudomonas aeruginosa bacteria, (B1) extract concentration of 4 mg/disk and 6 mg/disk, (B2) extract concentration of 8 mg/disk and 10 mg/disk against Staphylococcus aureus bacteria.

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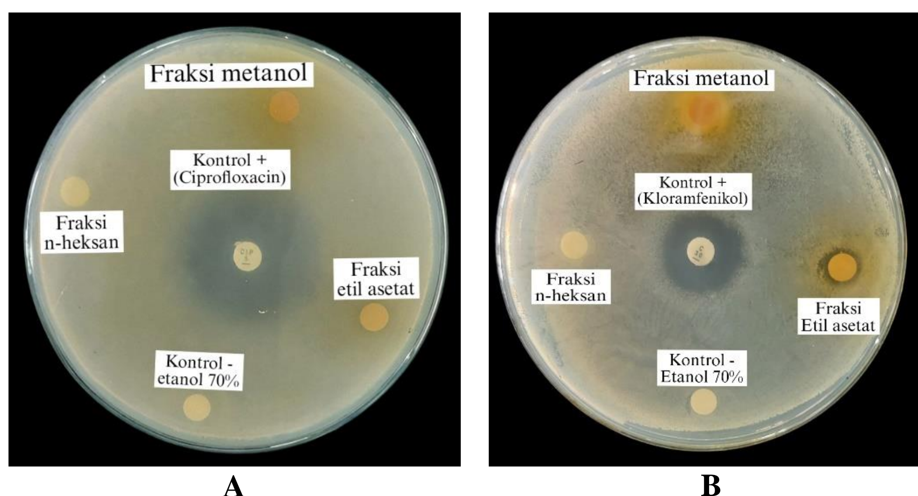

Figure 3. Results of the activity test of the seligi leaf fraction (Phyllanthus buxifolius muel. Arg) (A) n-hexane fraction, ethyl acetate fraction, 70% ethanol negative control and Ciprofloxacin positive control against Pseudomonas aeruginosa bacteria, (B) n-hexane fraction, ethyl acetate fraction, 70% ethanol negative control and chloramphenicol positive control against Staphylococcus aureus bacteria

Based on the test results in Table 3, seligi leaf extract did not show the formation of a clear zone against *Pseudomonas aeruginosa* bacteria at all concentrations tested. This is due to the lack of effective active compounds in the extract, in addition, the cell wall of Gram-negative bacteria is complex, consisting of peptidoglycan (inner layer), protein (outer layer), and lipopolysaccharides, making it difficult for antibacterial compounds to penetrate. Meanwhile, for *Staphylococcus aureus* bacteria, the largest inhibitory zone was formed at a concentration of 10 mg/disk with an average diameter of 12.16 ± 1.41 mm. The higher the concentration used, the greater the inhibition zone that is formed. This finding is consistent with the research of Raudah et al., (2020) who said that the higher the concentration of ethanol extract of seligi leaves (*Phyllanthus buxifolius Muell. Arg*) tested, the greater the diameter of the inhibition zone.

The results of the statistical analysis provided information that the testing data of seligi leaf extract (*Phyllanthus buxifolius Muell. Arg*) was analyzed using SPSS version 26 software. In the extract test on *Pseudomonas aeruginosa* bacteria, the normality and homogeneity tests obtained a significant value of 0.000 ($p < 0.05$), which showed that the data were not normally distributed and were not homogeneous. Then, the Kruskal-Wallis test was carried out which produced a significant value of 0.003 ($p < 0.05$). This result proves that there is a significant influence of the difference in seligi leaf extract on the inhibition zone formed. The Mann-Whitney test showed that there was no significant difference in all concentrations except for the positive control because of the significant value of 0.034 ($p<0.05$). In *Staphylococcus aureus* bacteria, the normality test obtained a significant value of 0.002, indicating that the data distribution was abnormal ($p > 0.05$), and the homogeneity test indicated a significant value of 0.047, indicating that the data were not homogeneous ($p > 0.05$). Thus, followed by the Kruskal-Wallis test which produced a significance value of 0.004 ($p < 0.05$), showing that there was a different effect of seligi leaf extract on the inhibition zone. The results of the Kruskal-Wallis test were followed by the Mann-Whitney test which proved that there was a significant difference in almost all concentrations, except for the 20% concentration group with a negative control that showed a significant 1,000 ($p > 0.05$). This is due to the absence of inhibition in

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this concentration, possibly because the content of active substances in the extract is not able to inhibit the growth of *Staphylococcus aureus bacteria*.

The results of the activity test on the fraction against *Pseudomonas aeruginosa* bacteria were not carried out statistical tests because there was no inhibition zone that formed in all solvents, except for the positive control of the inhibition zone which formed an average of 22.67 mm. In *Staphylococcus aureus bacteria,* statistical tests were also not carried out because there was no inhibitory zone formed in methanol and n-hexane solvents that formed only in the ethyl acetate fraction because it had a higher phenolic content than the methanol fraction and n-hexane fraction. Based on the test results, the extract and fraction of seligi leaves (*Phyllanthus buxifolius muell. Arg*) showed a greater inhibitory zone against *Staphylococcus aureus* bacteria (Gram-positive) than *Pseudomonas aeruginosa* bacteria (Gram-negative). This is caused by differences in the structure of the cell wall between Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a cell wall with a relatively thick layer of peptidoglycan, while Gram-negative bacteria have a more complex cell wall structure, consisting of peptidoglycan (inner layer), protein (outer layer), and lipopolysaccharides. Because of this difference, antibacterial compounds from seligi leaf extracts and fractions tend to penetrate more easily into the cell walls of Gram-positive bacteria than Gram-negative bacteria (Hamidah et al., 2019).

Phytochemical Test Results

Phytochemical testing is a method in identifying certain chemical compounds in a plant. This process includes testing compounds such as flavonoids, phenols, alkaloids, and terpenoids (Jafar et al., 2020). Thin-layer chromatography (KLT) is a method of separating compounds in extracts and fractions of lily leaves. This method has advantages because it is easy, simple, affordable, and takes a short time to operate. The KLT test is carried out using *a chamber* covered with filter paper, and saturation is carried out to equalize the steam pressure, so that the elution process can run well. The stationary phase used is GF254 silica gel plate, then the moving phase of the extract is a mixture of n-hexane: ethyl acetate with a ratio (6:4) (Mangunwardoyo et al., 2009). Seligi leaf extract is prepared with a concentration of 0.1% and placed in one spot of 1 microliter in each plate. The spray reagents used include citrorate, Dragendorff, FeCl3, and Liebermann-Burchard. In the ethyl acetate fraction, the phase of motion used is a mixture of ethyl acetate : methanol : formic acid with a ratio (6:4:1) (Mulqie & Anggadireja, 2020). The ethyl acetate fraction is prepared with a concentration of 0.1% and applied in one spot of 1 microliter on each plate. The spray reagents used for the ethyl acetate fraction are the same as those used in the KLT of lily leaf extracts, namely citrorate, Dragendorff, FeCl3, and Liebermann-Burchard. The Rf value obtained from KLT is used to identify the active compounds in the extract and fraction.

Table 2. results of phytochemical tests of KLT plates extract and ethyl acetate fraction of seligi leaves (*Phyllanthus buxifolius muell. Arg*)

Plate	Spray reagent	Rf	Visualization	Color/fluorescence	Information
70% ethanol extract	$FeCl_3$	0.84	Visible light	Black	+ phenolic
	Dragendorff	-	Visible light	Not orange brown	- alkaloids
	Sitroborat	0.7	UV366	Greenish yellow	+ flavonoids
	Liebermann-Burchard	0.84	UV366	Red or pink	+ Terpenoids
Ethyl acetate fraction	$FeCl_3$	0.6	Visible light	black	+ phenolic
	Dragendorff	0.88	Visible light	orange brown	+ Alkaloids
	Sitroborat	-	UV366	Not greenish yellow	- flavonoids
	Liebermann Burchard	-	UV366	Not red or pink	- Terpenoids

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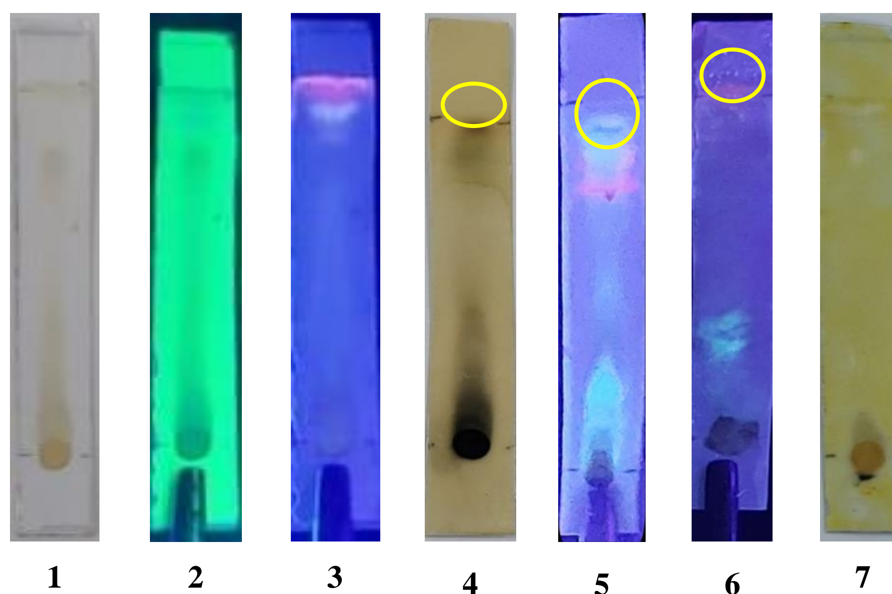

Figure 4. Phytochemical test results on KLT plates of 70% ethanol extract seligi (phyllanthus buxifolius muell. Arg), (1) KLT results in visible light, (2) KLT results at 254 nm UV light before spraying, (3) KLT results at 366 nm UV light before spraying, (4) FeCl3 reagent spraying results in visible light, (5) Citroborate reagent spraying results at UV 366 nm, (6) Lieberman-Burchard reagent spraying results at 366 nm UV light, (7) Dragendroff reagent spray results on visible rays.

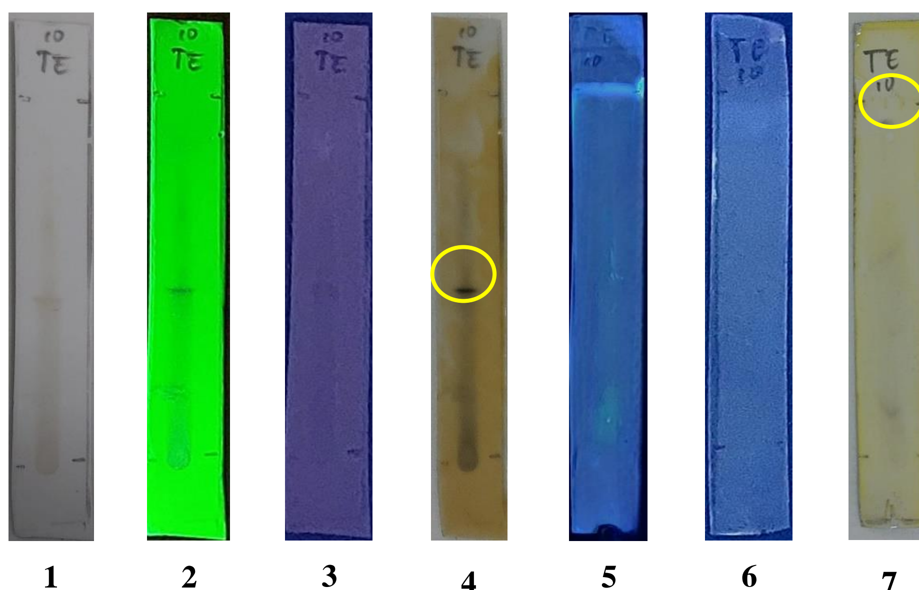

Figure 5 Results of phytochemical tests on KLT plates of seligi ethyl acetate fraction (Phyllanthus buxifolius muell. Arg), (1) KLT results on visible rays before spraying, (2) KLT results on 254 nm UV light before spraying, (3) KLT results on 366 nm UV light before spraying, (4) Results of FeCl3 reagent spraying on visible rays, (5) Citroborate regaen spray results at UV 366 nm, (6) Results of Lieberman-Burchard reagent spraying on 366 nm UV light, (7) Results of spraying Dragendroff reagent on visible light

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Identification of phenolic compounds using the FeCl3 reagent can produce spots that are black, blue, green, or red when exposed to visible light (Health, 2023). Seligi leaf extract showed positive results for phenolic compounds because it produced black spots with an Rf value of 0.84, while the ethyl acetate fraction was also detected to contain phenolic compounds with the appearance of black spots and had an Rf value of 0.6. Alkaloid compounds can be identified with the Dragendorff reagent which produces orange-brown or yellowish patches when exposed to visible light (Sunarmi & Suhendriyo, 2023). Seligi leaf extract does not show the presence of alkaloids because there are no characteristic orange-brown or yellowish spots in the visible light. The resulting patches tend to be thin or indecisive, indicating a possible low alkaloid content at that Rf value. On the other hand, the ethyl acetate fraction showed positive results for containing alkaloids because it produced clear orange-brown and yellowish spots, with an Rf value of 0.88. Flavonoid compounds can be identified by using citroborate reagents that produce greenish-yellow, orange, or red patches when exposed to 366 nm UV light (Harborne, 1987). Seligi leaf extract shows the presence of flavonoid compounds because it produces greenish-yellow and red patches with an Rf value of 0.7. However, in the ethyl acetate fraction, no greenish-yellow, orange, or red spots were observed when exposed to 366 nm UV light, suggesting the possibility that the levels of flavonoids escapsulated by the motion phase were still low at the Rf value. Thus, it is recommended to re-optimize the motion phase so that the results of the elution show more pronounced spots. The terpenoid compound class can be detected using the Liebermann-Burchard spray reagent, which produces patches that are red or pink in color when exposed to 366nm UV light (Tambunan et al., 2019). Seligi leaf extract showed the presence of terpenoids because it produced a red or pink spot with an Rf value of 0.84. However, in the ethyl acetate fraction, no red or pink spots were observed at 366 nm UV light. This is likely due to the number of terpenoids that have not been fully elucidated by the motion phase, so their concentration at the Rf value is quite low.

Hasil penelitian uji fitokimia daun seligi (*Phyllanthus buxifolius muell.Arg*) sejalan dengan penelitian Suhendriyo & Sunarmi (2023) bahwa ekstrak tidak mengandung alkaloid hal tersebut diduga dikarenakan terdapatnya faktor internal dan faktor eksternal yang bisa berpengaruh diantaranya seperti cahaya,suhu pH, dan ketinggian tempat hidup tetapi ekstrak positif mengandung fenolik, flavonoid dan terpenoid. Hasil penelitian Depkes (2000) menyatakan bahwa ekstrak daun seligi (*Phyllanthus buxifolius muell.Arg*) mengandung flavonoid, saponin dan polifenol. Hasil penelitian lain Amajida fildzah (2017) ekstrak daun seligi mengandung flavonoid, saponin, tannin dan alkaloid.

Bioautographic Test Results

Bioautography is a method that detects a chemical compound that can have an antibacterial role in seligi leaves (*Phyllanthus buxifolius muell. Arg*). The contact method test for KLT-Bioautographs is carried out by attaching KLT plates that have been marked with upper and lower limits as a sign of elucinations on the media that has been inoculated with the test bacteria. One of the drawbacks of the contact method is that it is difficult to achieve optimal contact between the KLT plates as well as the chromatogram so that the plates may stick or leave residues on the media with the aim of when the KLT plates are lifted again (Paputungan et al., 2019).

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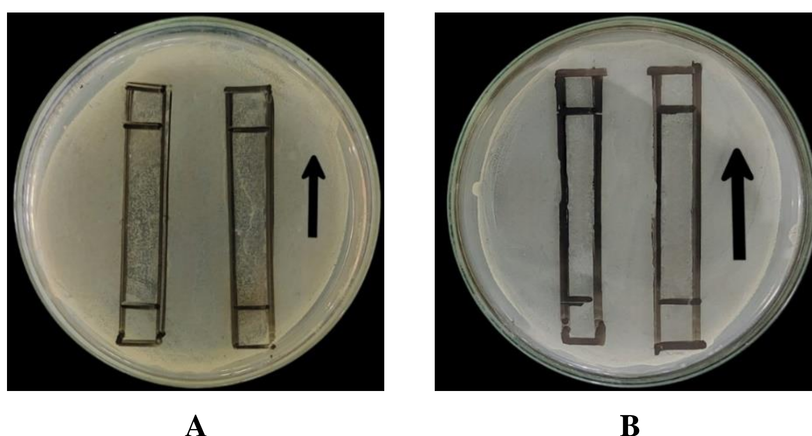

A B

Figure 6. KLT-Bioautography test results (A) lily leaf extract, (B) ethyl acetate fraction against Staphylococcus aureus bacteria

According to the results of bioautographic tests, no inhibitory zones were seen around RF 0.55, 0.35, 0.44, and 0.77 in *Staphylococcus aureus* bacteria. Meanwhile, in *Pseudomonas aeruginosa* bacteria, bioautographic tests were not carried out because no inhibitory zones were seen in the extract activity test. In the KLT test, it was identified that seligi leaf extract contains phenolic compounds because it can kill microorganisms by denatureizing cell proteins, so that all cell metabolic activities are catalyzed by enzymes, flavonoids because they damage the permeability of bacterial membranes and cell walls, and terpenoids because they form strong polymer bonds that reduce the permeability of bacterial cell walls which have the potential to be an obstacle to bacterial growth. However, in bioautographic testing, the compound was unable to grow bacteria, possibly due to the low concentration of the extract, which was 0.1%. These concentrations may not be sufficient to produce an inhibition in the growth of *Staphylococcus aureus* bacteria because the amount of active compounds in the extract is insufficient.

Conclusion

The results of the study proved that the extract and fraction of ethyl acetate from seligi leaves (Phyllanthus Buxifolius Muell.Arg) had antibacterial potential which was seen from the formation of an inhibitory zone in the antibacterial activity test against Staphylococcus aureus bacteria. However, in the bacterium Pseudomonas aeruginosa, no inhibitory zones were observed. The results of phytochemical tests indicate that seligi leaf extract contains phenolic compounds, flavonoids, and terpenoids, while the ethyl acetate fraction contains phenolic compounds and alkaloids.

Company

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