

Antibacterial activity of ethanolic and n-hexane extracts of *Ruellia tuberosa* leaves against *Escherichia coli* and *Bacillus subtilis* bacteria

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Abstract. Amijida H, Purwoko T, Susilowati A. 2019. Antibacterial activity of ethanolic and n-hexane extracts of *Ruellia tuberosa* leaves against *Escherichia coli* and *Bacillus subtilis* bacteria. *Biofarmasi J Nat Prod Biochem* 17: 69-80. *Ruellia tuberosa* L. has pharmacological activity, one of which is antibacterial. This study aimed to determine the antibacterial activity and the minimum inhibitory concentration (MIC) of pletekan leaf extract (*R. tuberosa*) against *Escherichia coli* and *Bacillus subtilis* and the chemical compounds contained in the leaves of *R. tuberosa*. This study used a completely randomized design with five treatments and three replications. *Ruellia tuberosa* leaf extract was obtained by extraction by maceration and reflux using ethanol and n-hexane as solvents. The concentrations of the *R. tuberosa* leaf extract used were: P1 (125 mg/mL), P2 (250 mg/mL), P3 (500 mg/mL), P4 (1000 mg/mL) and P5 (2000 mg/mL) with dimethylsulfoxide (DMSO) solvent. The positive control was in a 30 g chloramphenicol antibiotic disc, while the negative control used was DMSO solvent. The agar diffusion method used a paper disc to conduct the antibacterial activity test. The minimum inhibitory concentration of *R. tuberosa* leaf extract was determined using the dilution method. Identification of secondary metabolites was carried out using phytochemical screening. The data obtained were analyzed using the One-Way ANOVA test and if there was a significant difference, then continued with the Tukey test. *Ruellia tuberosa* leaf extract with ethanol solvent has better antibacterial activity against *E. coli* and *B. subtilis* than n-hexane. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *E. coli* was 500 mg/mL with a 99.1% inhibition percentage. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *B. subtilis* is 1,000 mg/mL with a 99% inhibition percentage. The ethanolic extract of *R. tuberosa* leaves contains alkaloids, flavonoids, tannins, saponins, and glycosides, while the n-hexane extract of *R. tuberosa* leaves contains terpenoids.

Keywords: Antibacterial, *Bacillus subtilis*, *Escherichia coli*, phytochemical screening, *Ruellia tuberosa*

INTRODUCTION

Antibacterial is a substance that inhibits or kills the growth of microorganisms. Microorganism growth control aims to prevent disease and infection from spreading, eradicate microorganisms in infected hosts, and prevent spoilage and destruction of materials by microorganisms (Setyaningsih et al., 2012). Antibacterial power was measured in vitro to determine the ability of an antibacterial substance. The phenomenon of natural plant resistance to bacteria has led to the development of several compounds derived from plants with antibacterial and antifungal properties (Jawetz et al., 2008).

Chemical compounds resulting from secondary metabolites have been widely used as dyes, poisons, food aromas, and medicines. Various plants are used as traditional medicines, so research is needed on efficacious plants and the chemical compounds that function as drugs (Afristiani and Astuti 2005; Lenny 2006). A solvent can pull the compounds' content in plants during the extraction process. Selecting a suitable solvent is an essential factor in the extraction process. The solvent used in the extraction determines the quality of the simplicia, namely the composition of the compounds contained in the extract. Ethanol is often used as a solvent in the laboratory because it has relatively high solubility and is inert, so it does not

react with other components. Ethanol is a polar solvent, so the active components contained in plants can be extracted more completely. Meanwhile, n-hexane is the lightest solvent in the extraction process and evaporates quickly, making it easier for reflux (Susanti et al., 2012).

Based on the cell wall composition, bacteria are divided into two, namely Gram-positive and Gram-negative bacteria. Gram-positive and Gram-negative bacteria cell walls are composed of a peptidoglycan polymer with elastic peptide-glycan cross-links. The resulting elastic tissue protects the cell from lysis. Gram-positive bacteria have a thick layer of peptidoglycan, while Gram-negative bacteria have a thin layer of peptidoglycan but have an additional membrane, namely the outer cytoplasmic membrane. This outer membrane layer functions as an additional permeability barrier and is used as a mechanical transport membrane (Pelczar and Chan 2008).

Ruellia tuberosa L. is traditionally used to treat diuresis, antidiabetic, antipyretic, antihypertensive, and antidote agents. In Taiwan, *R. tuberosa* is a simplicia added to health drinks (Lin et al. 2006). In Trinidad and Tobago, *R. tuberosa* is used as an antihypertensive and cooling agent (Lans 2006). This plant has not been widely used in Indonesia; even better known as a weed. Increasing its use requires scientific studies regarding this plant's biological or pharmacological activities (Ahmad 2012).

Several chemical constituents and antimicrobial activity have been reported for *R. tuberosa* from Taiwan and India. Chloroform, ethyl acetate, alcohol, and aquadest extracts from the whole plant *R. tuberosa* showed significant antibacterial properties. The distilled water extract showed less activity against fungal organisms (Ariudran et al., 2011).

The methanolic extract of *R. tuberosa* root showed significant antibacterial and antifungal properties with an inhibition zone of 9-23 mm for antibacterial screening and 8-15 mm for antifungal screening. Tests of insecticides using the surface film activity test showed strong insecticidal activity with a mortality rate of 80% of *Tribolium castaneum* (Herbst) at a 50 mg/mL dose in 48 hours (Kader et al. 2012).

The methanolic extract of the leaves of *R. tuberosa* showed significant antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Proteus mirabilis*, and antifungal activity against *Aspergillus* sp., *Mucor* sp., *Penicillium* sp. and *Fusarium* sp. The antibacterial potential of the methanolic extract of *R. tuberosa* was tested using the agar diffusion method. A total of 100 mg/mL of leaf extract showed the maximum zone of inhibition against *P. mirabilis* (7mm) and *Aspergillus* sp. (8 mm) (Vasantharaj et al., 2013). From the literature review, it is known that *R. tuberosa* has antibacterial activity. Therefore, this study was conducted to increase its utilization to determine the power of antibacterial leaf extract of *R. tuberosa* against the growth of *E. coli* and *B. subtilis*.

The aims of this study were: (i) To determine the antibacterial activity of pletekan leaf extract (*R. tuberosa*) with ethanol and n-hexane as solvents against *E. coli* and *B. subtilis*. (ii) Knowing the minimum inhibitory concentration (MIC) of *R. tuberosa* leaf extract with ethanol and n-hexane as solvents against *E. coli* and *B. subtilis*. (iii) Knowing the chemical compounds contained in the leaf extract of *R. tuberosa* with ethanol and n-hexane as solvents.

MATERIALS AND METHODS

Research time and place

The study held from December 2015 to March 2016 took place at the Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret (UNS), Surakarta, Sub-laboratory Chemistry UNS Surakarta, Central Java, Indonesia, and Laboratory of Food and Nutrition Culture Collection (FNCC) Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia.

Ingredient

The materials used in this study included: isolates of *E. coli* bacteria IFO 3301, *B. subtilis* bacterial isolate IFO 13719, and leaves of *R. tuberosa* obtained from Surakarta (Solo), Central Java, Indonesia.

Research design

This research uses a completely randomized design (CRD) with five treatments, namely variations in the concentration of *R. tuberosa* leaf extract with ethanol and n-hexane solvent, positive control in the form of chloramphenicol antibiotic disc 30 ug, and the negative control was DMSO solvent.

Procedures

Material preparation

The fresh leaves of the *R. tuberosa* plant were washed with running water until clean, drained, and air-dried so that they were free of the remaining water. *R. tuberosa* leaves were blended for ± 2 minutes so that the samples of *R. tuberosa* leaves were smooth.

Sterilization

The equipment to be used must be sterilized first to avoid contamination. The sterilized equipment includes Petri dishes, Erlenmeyer, test tubes, beakers, stirring rods, and ose needle. Materials that must be sterilized are Nutrient Agar media and Mueller Hinton media. Sterilization is carried out using an autoclave at a temperature of 121°C, a pressure of 1 atm for 15 minutes.

Extraction

The leaves of the *R. tuberosa* plant were cleaned and then aerated to dry, with a moisture content of 5%. The dried leaves are cut into pieces and blended until a powder is formed. A total of 300 g of powder in a 2,000 mL Erlenmeyer was macerated in 96% ethanol until all the powder was submerged (1,500 mL). Maceration was carried out for 6 hours while shaking using a shaker at 40 rpm. The soaked leaf powder of *R. tuberosa* was refluxed for 3 hours and filtered using Whatman No. filter paper. 42. The filtering dregs were refluxed with 96% ethanol and repeated two times. The ethanol contained in the filtrate was removed by evaporation using a vacuum evaporator at 40°C so that a thick extract of 96% ethanol was obtained. The ethanol dregs were extracted again using n-hexane by refluxing two times and filtered using Whatman filter paper No.42. The resulting filtrate was removed by evaporation using a vacuum evaporator at 40°C to obtain a thick extract of n-hexane. The yields obtained were weighed and recorded (Age et al. 2002; Harborne 2006).

$$\% \text{ extract content} = \frac{\text{extract weight obtained (g)}}{\text{extracted sample weight (g)}} \times 100\%$$

Extract moisture test

The water content test was carried out to determine the remaining water in the extract. The procedure for testing the moisture content of the extract was as follows: 1 g of ethanolic extract and n-hexane were put into a porcelain dish that had been tared separately, then dried in an oven at 105°C for 5 hours, followed by drying at a distance of 1 hour until the difference between 2 weights in a row, not more than 0.25% (Indonesian Ministry of Health 2000).

$$\% \text{ drying shrink} = \frac{b-c}{b-a} \times 100\%$$

Where:

a = weight of the empty cup

b = weight of sample and cup before drying in the oven

c = weight of sample and cup after drying in the oven

Media preparation

Nutrient agar powder was weighed as much as 2.3 g and mixed with 100 mL of distilled water in an Erlenmeyer, then heated and stirred using a stirrer on a hotplate until dissolved. With the same treatment, 0.8 g of Nutrient Broth (NB) was mixed with 100 mL of distilled water in an Erlenmeyer, then heated and stirred using a stirrer on a hotplate until dissolved.

Preparation of test extract concentration

In this study, the concentration series of the test extracts (ethanol and n-hexane) used were 2000, 1000, 500, 250, and 125 mg/mL with dimethylsulfoxide (DMSO) solvent. The 2000 mg/mL test extract was prepared by weighing 400 mg of the extract and dissolved in 0.2 mL of DMSO. Concentrations of 1000, 500, 250, and 125 mg/mL were made by serial diluting the extract with DMSO. Sterile disc paper was saturated with 10 L of the test extract solution and dried in a sterile petri dish at room temperature.

Positive control and negative control

The positive control used was chloramphenicol antibiotic disc 30 g, while the negative control used was DMSO solvent (Natheer et al. 2012).

Bacteria suspension manufacturing

One ose of bacterial colonies was inoculated in 10 mL of NB and then incubated at 37°C for 24 hours. The optical density of the culture was measured using a spectrophotometer at a wavelength of 625 nm (OD₆₂₅). The resulting OD₆₂₅ is then converted to 0.1. OD₆₂₅ 0.1 is equivalent to 0.5 McFarland standard (bacterial cell density 1x10⁸ cells/mL). The bacterial suspension was then diluted into a bacterial suspension with a cell density of 10⁶ cells/mL by putting 1 mL of a 108 cell/mL bacterial suspension into a test tube containing 9 mL. NB was then vortexed, and a bacterial suspension was produced with a cell density of 10⁷ cells/mL. Then 1 mL of bacterial suspension of 10⁷ cells/mL was put into a test tube containing 9 mL of NB, vortexed, and a bacterial suspension with a cell density of 10⁶ cells/mL was produced (Lopez et al. 2003; Widiastomo and Wahyu 2013).

Antibacterial test procedure

A total of 1 mL of bacterial suspension with a cell density of 10⁶ cells/mL was poured into 15 ml of Nutrient agar that had solidified, then leveled using a glass rod. Preparation of media and pouring of bacterial suspension was carried out near the bunsen burner in Laminar Air Flow. After the agar solidified, each petri dish was made a

7-part diagram. Positive control paper discs, negative control discs, and discs saturated with extract solution were placed in each section and then incubated at 37°C for 24 hours (Nufailah et al. 2008). This antibacterial test was repeated three times. The clear area around the disc indicated the inhibition of bacterial growth, which was then measured using a caliper. The diameter and area of the inhibition zone were calculated.

The formula calculates the diameter of the inhibition zone (D):

D = Inhibitory zone diameter – paper disc diameter

$$r = \frac{D}{2}$$

Furthermore, the area of the inhibition zone (L) was calculated using the formula:

$$L = \pi r^2$$

π = constant

r = radius of inhibition zone (mm)

The results of the inhibition zone measurements were classified according to Table 1.

Minimum Inhibitory Concentration (MIC) of *R. tuberosa* leaf extract was determined by tube dilution test by decreasing the extract concentration (to close the dose) based on the diameter of the inhibition zone formed in the diffusion test. A total of 4 mL of sterile NB media was put into each test tube, added by extract according to the concentration of 0.5 mL. Subsequently, 0.5 mL of bacterial suspension was added to this medium at 10⁶ cells/mL, adjusted to the 0.5 Mc standard Farland (repeated three times). Then the tubes were incubated for 18-24 hours at 37°C in an incubator (Fatisa 2013).

All variations in the concentration of the extract were compared with bacterial control and media control by looking at the turbidity of the test sample. The concentration of *R. tuberosa* leaf extract against bacteria showing clear color was then replanted on NB media to determine if the extract was bacteriostatic or bactericidal.

Data analysis of the liquid dilution method results was carried out by calculating the minimum inhibitory concentration value. The formula used to calculate the inhibition of bacterial growth is as follows (Quave et al. 2008):

$$\% \text{ inhibition} = \left[\frac{\text{sample OD} - \text{blank sample OD}}{\text{DMSO OD} - \text{blank DMSO OD}} \right] \times 100\%$$

Where:

Sample OD: Optical density extract with suspension

Blank sample OD: Optical density extract with saline

DMSO OD: Optical density DMSO with microbial suspension

Blank DMSO OD: Optical density DMSO with saline

Phytochemical screening

Phytochemical screening was carried out on ethanol and *n*-hexane extracts. This test was conducted to determine the secondary metabolites of alkaloids, flavonoids, tannins, terpenoids, saponins, and glycosides. The test procedure is as indicated in Table 2.

The expansion vessel is saturated with the appropriate mobile phase for each class of active compound. The thick extract of the leaves of *R. tuberosa* was spotted on the layer chromatography (TLC) plate, and then the TLC plate was immersed in the developer's vessel. After that, the TLC plate was eluted, dried, and then detected with the appropriate spectrophotometer for each group of compounds present in the thick extract of *R. tuberosa* leaves. It was then observed in visible light and calculated its R_f (Wagner et al. 1984).

Data analysis

The diameter of the clear zone around the paper disc in each extract was analyzed descriptively with the help of tables and figures. The analysis used is a one-way ANOVA statistical test. One way ANOVA test was used to determine the effect of giving various concentrations of *R. tuberosa* leaf extract on the growth of *E. coli* bacteria and *B. subtilis*. If there is a difference, it is continued using the Tukey test.

RESULTS AND DISCUSSIONS

Ruellia tuberosa leaf extraction with ethanol and *n*-hexane solvents

The technique of isolating secondary metabolites from natural materials is known as extraction. *R. tuberosa* leaves were extracted by maceration and reflux to extract the chemical components contained in *Simplicia* using ethanol and *n*-hexane as solvents. Each character, weight, and water content of the extract is presented in Table 3.

Based on the general standard parameters of plant extracts from the Ministry of Health of the Republic of Indonesia (2000), the water content limit of the extract is <10%. It indicates that the water content of the ethanol extract and *n*-hexane corresponds to the standard parameters of the extract water content. The quality of extracts produced from plant materials largely depends on the solvent used in the extraction procedure. Good solvent properties in plant extraction include low toxicity, volatility at low temperatures, and the ability to dissolve bioactive compounds contained in natural ingredients (Anand et al., 2015).

Table 1. Classification of antibacterial activity (Greenwood 1995)

Inhibition zone diameter (mm)	Antibacterial activity
10	Not active
11-15	Weak
16-20	Currently
20	Strong

Table 3. Character *R. tuberosa* leaf extract with ethanol and *n*-hexane as solvent.

Extract	Ethanol	<i>n</i> -Hexane
Character		
Form	Thick extract	Thick extract
Color	Dark chocolate	blackish green
Smell	Typical	Typical
Extract weight		
Weight (grams)	30.35	4.89
Yield (%)	10,12	1.63
Water content		
Initial weight (g)	20, 3333	20.9407
Final weight (g)	20.2742	20.8719
Water content (%)	5.74	7.16

Note: Initial weight: The weight of the sample and crucible before drying in the oven; Final weight: Weight of sample and cup after drying in the oven

Table 2. Chromatographic characteristics

	Alkaloids	Flavonoids	Tannins	Terpenoids	Saponins	Glycoside
Silent phase	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254
Mobile phase	Toluene: ethylacetate: diethylamine (7:2:1)	Ethylacetic: formic acid: acetic acid glassial: water(100:11:11:27)	chloroform: ethylacetate: formic acid (0.5:9:0.5)	Hexane: ethylacetate (93:7)	Chloroform: methanol: water (64:50:1)	Ethylacetic: formic acid: toluene: water (6:1.5:3:0.5)
Comparison	Quinine 10 mg/ 1 mL ethanol	Routine 10 mg/1 mL ethanol	Tannins 10 mg/ 1 mL ethanol	Tymol 10 mg/1 mL ethanol	Saponins 10 mg/1 mL ethanol	Gallic acid 10 mg/1 mL ethanol
Detection	Dragendor (viewed after sprayed on visible light orange)	cytroborate (viewed after sprayed on visible light-colored yellow)	FeCl ₃ (viewed after sprayed on the light looks colorful dark green-black)	Anisaldehyde sulfuric acid (viewed after being sprayed on the light looks colored Red purplish)	Lieberman Bourchat (viewed after sprayed on visible light (blackish brown))	FeCl ₃ (seen after being sprayed on visible light is dark green-black)

Antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane

Antibacterial is a typical chemical compound produced by living organisms in low concentrations and can inhibit essential processes in a microorganism. Disc diffusion test is carried out by measuring the diameter of the clear zone, which indicates a response to inhibition of bacterial growth by an antibacterial compound in the extract (Hermawan et al. 2007). The antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane as solvents against *E. coli* and *B. subtilis* bacteria can be seen in Figure 3.

Table 4 shows that the ethanolic extract of the leaves of *R. tuberosa* against *E. coli* had a larger diameter of inhibition zone than *B. subtilis*. Based on the classification of antibacterial activity by Greenwood (1995), a concentration of 2000 mg/mL of ethanolic extract of *R. tuberosa* leaves against *E. coli* had weak antibacterial activity. On the other hand, it had no antibacterial activity or was inactive for other concentrations because the diameter of the inhibition zone was 10 mm. The inhibition zone diameter formed in the ethanol extract of the leaves of *R. tuberosa* against *B. subtilis* did not show any antibacterial activity because the inhibition zone diameter was 10 mm.

Table 5 shows only the concentration of 2,000 mg/mL of n-hexane leaf extract against *E. coli* which resulted in

the presence of an inhibition zone, while for *B. subtilis* at all variations of extract concentration did not produce an inhibition zone. Based on the classification of antibacterial activity by Greenwood (1995), n-hexane extract against *E. coli* and *B. subtilis* did not have an antibacterial activity or was inactive because the diameter of the inhibition zone was 10 mm. Based on Table 4 and Table 5, the comparison of the diameter of the inhibitory zone of *R. tuberosa* leaf extract with ethanol and n-hexane solvents against *E. coli* bacteria and *B. subtilis* can be seen on the next curve (Figure 4A-D).

Figure 4 shows the diameter of the inhibition zone of the ethanolic extract of the leaves of *R. tuberosa* against *E. coli* was greater than that of *B. subtilis*, which means that the ethanol extract had more significant antibacterial activity against *E. coli* than *B. subtilis*. Meanwhile, the n-hexane extract of *B. subtilis* did not show any inhibition zone, in contrast to *E. coli* which only shows the diameter of the inhibition zone at a concentration of 2000 mg/mL (Figure 4B). Figure 4C and 4D show that the diameter of the inhibition zone of the ethanolic extract of *R. tuberosa* leaves against *E. coli* and *B. subtilis* was greater than that of the n-hexane extract. It means that the ethanol extract had more significant antibacterial activity than n-hexane extract in inhibiting the growth of *E. coli*. and *B. subtilis*.

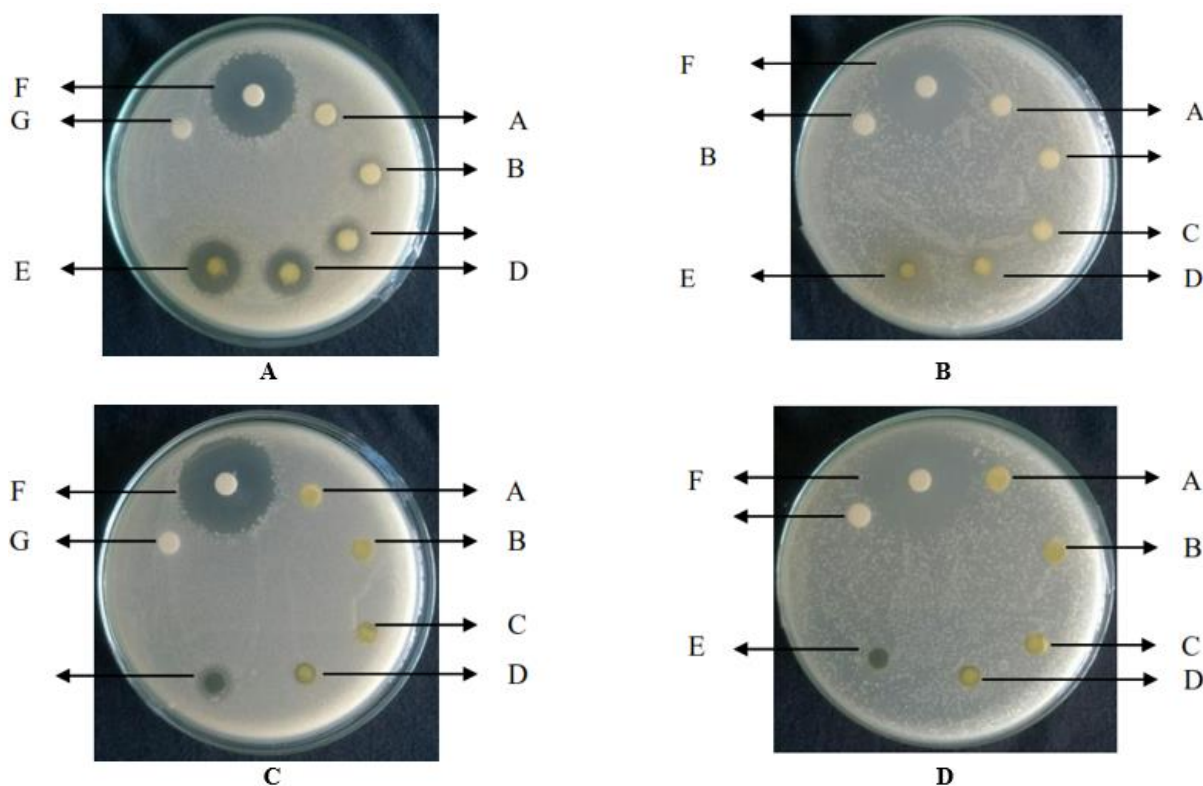


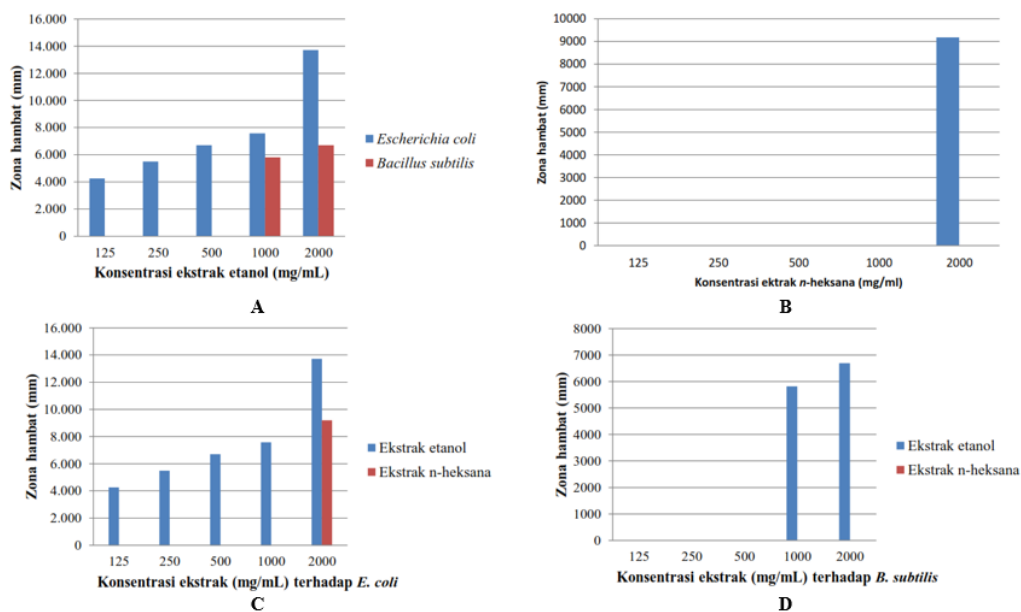
Figure 3. A. Diameter resistor extracts ethanol leaf *R. tuberosa* to bacteria *E. coli* (A) and *B. subtilis* (B). Inhibitory diameter of n-hexane extract of leaves of *R. tuberosa* to bacteria *E. coli* (C) and *B. subtilis* (D). Description: A. Concentration 125 mg/mL, B. Concentration 250 mg/mL, C. Concentration 500 mg/mL, D. Concentration 1000 mg/mL, E. Concentration 2000 mg/mL, F. Positive control, G. Negative control

Table 4. Diameter and wide zone resistor extract ethanol leaf *R. tuberosa* against bacteria *E. coli* and *B. subtilis*

Concentration extract	<i>Escherichia coli</i>		<i>Bacillus subtilis</i>	
	Diameter(mm)	Area (mm ²)	Diameter(mm)	Area (mm ²)
2000 mg/mL	13.706	147,466	6,696	35,196
1000 mg/mL	7.569	44,972	5,814	26,535
500 mg/mL	6,702	35,259	0	0
250 mg/mL	5,488	23,643	0	0
125 mg/mL	4,253	14,199	0	0
positive control	18,258	261,683	9,586	72.135
Negative control	0	0	0	0

Table 5. Diameter and area of inhibition zone of *R. tuberosa* leaf *n-hexane* extract against *Escherichia coli* and *Bacillus subtilis* bacteria

Concentration extract	<i>Escherichia coli</i>		<i>Bacillus subtilis</i>	
	Diameter(mm)	Area (mm ²)	Diameter(mm)	Area (mm ²)
2000 mg/mL	9.181	66,154	0	0
1000 mg/mL	0	0	0	0
500 mg/mL	0	0	0	0
250 mg/mL	0	0	0	0
125 mg/mL	0	0	0	0
positive control	18,394	265.596	11.450	102,915
Negative control	0	0	0	0

**Figure 4.** A. Antibacterial activity of *R. tuberosa* leaf ethanol extract against bacteria *E. coli* and *B. subtilis*; B. Antibacterial activity of n-hexane extract of *R. tuberosa* leaves against bacteria *E. coli* and *B. subtilis*; C. Antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane against *E. coli* bacteria; D. Antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane against the bacterium *B. subtilis*.**Table 6.** Tukey analysis of inhibition of ethanolic extract of *R. tuberosa* leaves against *Escherichia coli* bacteria.

Variation of extract concentration	Inhibition zone diameter (mm)
125 mg/mL	4.25300 ^a
250 mg/mL	5,48767 ^b
500 mg/mL	6.70233 ^c
1000 mg/mL	7.56967 ^c
2000 mg/mL	13,70567 ^d
positive control	18,25833 ^e

Note: letters a, b, c, d, and e are significant values indicating a significant difference between each treatment

Table 7. Tukey's inhibition of ethanolic extract of *R. tuberosa* leaves against *Bacillus subtilis* bacteria.

Variation of extract concentration	Inhibition zone diameter (mm)
1000 mg/mL	5,81367 ^a
2000 mg/mL	6,69633 ^{ab}
positive control	9.58600 ^b

Note: letters a, b, c, d, and e are significant values indicating a significant difference between treatments

One-Way ANOVA statistical analysis to see if there are significant differences in the overall treatment. The results of the One-Way Anova test show that $p = 0.000$, so it can be interpreted that there is a significant difference between the five treatment groups above because $p < 0.05$. Because there are differences, it is continued by using the Tukey test, which can be seen in Table 6 and Table 7.

Tukey's statistical analysis in Table 6 shows that the inhibitory power of the ethanol extract of the leaves of *R. tuberosa* at a concentration of 2000 mg/mL is close to the positive control in inhibiting the growth of *E. coli*. Meanwhile, the 125 mg/mL concentration was at least close to the positive control in inhibiting the growth of *E. coli*. Tukey's statistical analysis in Table 7 shows that the inhibitory power of the ethanol extract of the leaves of *R. tuberosa* at a concentration of 2000 mg/mL is close to the positive control in inhibiting the growth of *B. subtilis*. At the same time, the concentration of 1000 mg/mL did not approach the positive control in inhibiting the growth of *B. subtilis*.

In general, the inhibition diameter data in Table 4 and Table 5 showed that the ethanolic extract of *R. tuberosa* leaves had a greater inhibitory power on bacterial growth than the n-hexane extract of *R. tuberosa* leaves. It is due to the presence of secondary metabolites in the test extract. The results of the phytochemical screening test showed that the ethanolic extract of the leaves of *R. tuberosa* contained alkaloids, flavonoids, tannins, saponins, and glycosides. The content of these compounds is more complex than the n-hexane extract of *R. tuberosa* leaves which only contains terpenoid compounds. The more active compounds in the extract, the greater the antibacterial activity. According to Kavitha et al. (2012), an increase in the concentration of the extract causes an increase in the content of active compounds that function as antibacterial so that the antibacterial activity is more significant.

Okeke et al. (2001) and Rahman et al. (2011) stated several secondary metabolites such as glycosides, saponins, tannins, flavonoids, terpenoids, and alkaloids had been reported to have antibacterial activity. Plants synthesize flavone compounds, flavonoids, and flavonols to respond to microbial infections (Ncube et al. 2008). Anyasor et al. (2011) stated that the antibacterial activity of plant extracts might be due to the presence of tannins and flavonoids compounds that bind to the bacterial cell wall and inhibit their biosynthesis.

Flavonoids are synthesized by the plant to respond to infection bacteria and have antibacterial activity in vitro against various pathogenetic. Flavonoids can shape bond complex with extracellular protein and dissolve with Wall cell bacteria, pushing disturbance membrane cell microbes (Moyo et al. 2012). The assembly process of microbial cell walls begins with forming peptide chains that will create peptide cross bridges and combine the glycan chains of peptidoglycan in other chains, causing the cell wall to be perfectly assembled. Suppose there is damage to the cell wall or obstacles in its formation. In that case, lysis of microbial cells can occur so that microbes immediately lose the ability to form colonies and are followed by cell death microbes. Flavonoid compounds inhibit cell wall assembly

and incorporate glycan chains not connected to the cell wall peptidoglycan into a weak structure and cause microbial death (Ajizah 2004).

Alkaloids are the largest group of secondary plant substances. Alkaloids have the ability to an antibacterial. The mechanism is by interfering with the peptidoglycan constituent components in bacterial cells so that the cell wall layer is not fully formed and causes cell death (Darsana et al. 2012). The mechanism of bacterial cell wall damage occurs due to alkaloids having primary groups that will come into contact with the amino acids that make up the tetrapeptide which will form cross-bridges in the synthesis of bacterial cell walls. The bond between alkaloids and amino acids results in cross bridges that cause stiffness in the bacterial cell wall not to form. This situation causes bacterial cells to undergo lysis easily, either under physical or osmotic pressure, and causes cell death (Ajizah 2004).

Tannins are polyphenolic compounds that are soluble in water and polar solvents. Tannin compounds can inhibit and kill bacterial growth by reacting with cell membranes. Astringent tannin compounds can induce the formation of complex compounds with bacterial proteins. The bond between astringent compounds and bacterial cell wall proteins causes bacterial proteins to be denatured. When disturbed, proteins that are components of enzymes will interfere with enzyme activity so that the cell walls are formed fragile. As a result, the cell wall becomes weak, and the cell will break or lyse so that the bacteria will die (Akiyama et al., 2001; Moyo et al., 2012).

Saponins work as antibacterial by damaging the cytoplasmic membrane. Damage to the cytoplasmic membrane can reduce the permeability of the cell membrane so that the transport of substances into and out of the cell becomes uncontrolled. Substances inside the cell, such as organic ions, enzymes, amino acids, and nutrients, can leave the cell. If the enzymes out of the cell together with substances such as water and nutrients can cause metabolism to be inhibited, resulting in a decrease in ATP required for cell growth and reproduction, so that bacterial cell growth is inhibited and causes cell death (Hertiani et al. 2003; Rahmawati et al. 2014).

Mechanism terpenoids, as an antibacterial, react with Porin (protein transmembrane) on the membrane outside Wall cell bacteria, shape bond strong polymer, resulting in the damage Porin (Cowan 1999). Terpenes and terpenoids are active in bacteria, fungi, viruses, and protozoa (Enwa et al., 2014).

Chloramphenicol is used as positive control by inhibiting protein synthesis in bacterial cells. Chloramphenicol will bind reversibly to the 50S ribosomal unit, preventing the binding of amino acids to the ribosome. This antibiotic binds specifically to the acceptor (initial binding site of aminoacyl-tRNA) or the peptidyl site, a critical binding site for peptide chain elongation (Katzung 2004).

Besides maximizing the potential of Indonesia's natural resources, which are rich in flora, the development of knowledge about medicinal plants can also minimize the side effects often caused by modern medicines. The

metabolic processes found in plants are primary and secondary metabolism. Primary metabolic processes produce compounds used in daily biosynthesis processes: carbohydrates, proteins, fats, and nucleic acids. On the other hand, secondary metabolic processes produce compounds with certain biological activities, such as alkaloids, terpenoids, flavonoids, tannins, and steroids. Sometimes the compounds contained by a plant of a particular genus are specific. For example, plants from the genus *Papaver*, *Papaver somniferum*, and *Papaver septigerum* produce *morphine* and have calming properties (Hanani 2010).

The function of the first secondary metabolites is to protect plants from microbial attack. For example, plants form phytoalexins, unique compounds synthesized around infected cells. Second, to defend themselves from predators. Third, to fight herbivores' disturbances by creating toxic compounds that cause them to become toxic. Fourth is protecting the environment; for example, anthocyanins are produced to protect plants from UV rays. Fifth, win the competition by producing allelopathic compounds, which are toxic to other plants in the vicinity. Sixth, as an attractant agent, it attracts insects and other herbivores to help disperse seeds. The compound is a

pigment that gives the reproductive organs a bright color. It is impossible to contain one kind of secondary metabolite inside one Plants. Certain plants can have several different kinds of therapeutic properties according to the secondary metabolites contained (Hanani 2010).

***Ruellia tuberosa* leaf extract with solvent ethanol and n-hexane**

Based on the antibacterial test, it was found that the ethanolic extract of the leaves of *R. tuberosa* gave different antibacterial activity against the two test bacteria compared to the n-hexane extract of the leaves of *R. tuberosa*. Therefore, in determining the minimum inhibitory concentration, the ethanol extract of the leaves of *R. tuberosa* against *E. coli* was used with a test solution concentration of 500; 250; 125; 62.5; 31.25, and 15.625 mg/mL, as well as the concentration of the test solution 1000; 500; 250; 125; 62.5 and 31.25 mg/mL for the ethanolic extract of the leaves of *R. tuberosa* against *B. subtilis*. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *E. coli* and *B. subtilis* can be seen in Figures 5 and Figure 6.

Table 8. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *E. coli* and bacteria *B. subtilis*

Concentration extract	OD			% Inhibition	KHM
	I	II	III		
<i>Escherichia coli</i>					
500 mg/mL	0.662	0.580	0.611	99.1	500 mg/mL
250 mg/mL	0.150	0.147	0.148	99	
125 mg/mL	0.283	0.219	0.244	81.1	
62.5 mg/mL	0.202	0.275	0.261	80.9	
31.25 mg/mL	0.630	0.599	0.528	23.4	
15.625 mg/mL	0.606	0.609	0.619	15.4	
<i>Bacillus subtilis</i>					
1000 mg/mL	0.665	0.654	0.654	99	1000 mg/mL
500 mg/mL	0.388	0.380	0.383	82.8	
250 mg/mL	0.545	0.548	0.548	67.8	
125 mg/mL	0.614	0.615	0.615	59.4	
62.5 mg/mL	1.059	1.058	1.061	20.4	
31.25 mg/mL	1,246	1,247	1,248	2.8	

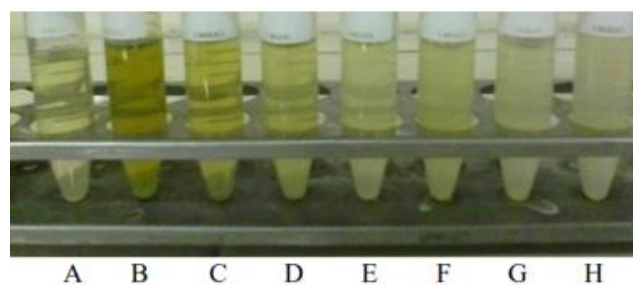


Figure 5. The level of turbidity produced by the ethanol extract of the leaves of *R. tuberosa* against *Escherichia coli* bacteria. A. Control media, B. Concentration 500 mg/mL, C. 250 mg/mL, D. 125 mg/mL, E. 62.5 mg/mL, F. 31.25 mg/mL, G. 15.625 mg/mL, H. Control bacteria

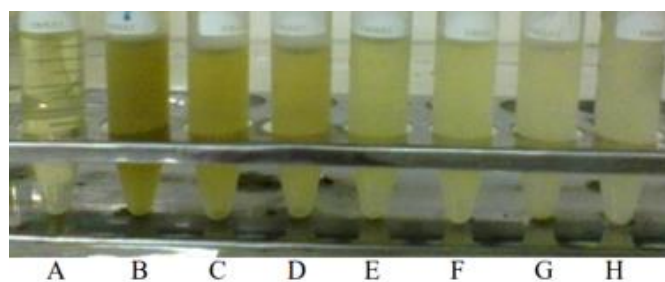


Figure 6. The level of turbidity produced by the ethanol extract of the leaves of *R. tuberosa* against *Bacillus subtilis* bacteria. A. Control media, B. Concentration 500 mg/mL, C. 250 mg/mL, D. 125 mg/mL, E. 62.5 mg/mL, F. 31.25 mg/mL, G. 15.625 mg/mL, H. Control bacteria

From Table 8, it can be seen that the value of the minimum inhibitory concentration for the ethanolic extract of the leaves of *R. tuberosa* against *E. coli* was 500 mg/mL with an inhibition percentage of 99.1%. In comparison, the MIC value for the ethanol extract of the leaves of *R. tuberosa* against *B. subtilis* was 1000 mg/mL with an inhibition percentage of 99%.

The MIC value is expressed as the lowest concentration of extract that can still inhibit the growth of the test bacteria (Pratiwi 2008). The antibacterial MIC value varies depending on the type of bacteria and the antibacterial compounds contained in it. The results of the research showed that the smaller the test concentration, which means the less amount of active substance dissolved in the extract, the lower the ability of the test material to inhibit the growth of a bacterium.

There is a difference in the inhibitory power of the ethanolic extract of the leaves of *R. tuberosa* against the bacteria *E. coli* and *B. subtilis*. The cell walls of Gram-positive bacteria are composed of several layers of peptidoglycan, forming a thick and rigid structure. The thick and rigid cell wall structure makes Gram-positive bacteria resistant to lysis caused by osmotic pressure. On the other hand, Gram-negative bacteria consist of one or very few layers of peptidoglycan in their cell walls. In addition, the cell walls of Gram-negative bacteria contain no teichoic acids but some polysaccharides, making them

more susceptible to mechanical and chemical damage (Jawetz et al. 2008).

The glycan chain in *E. coli* is not cross-linked and may be free of part or all tetrapeptide units. Therefore, the cross-linking of peptidoglycan in Gram-positive bacteria is only about 30-70%. In contrast, glycans in Gram-positive bacteria retain all tetrapeptide units completely cross-linked. In addition to variations in cross-linking, variation occurs in the presence of polypeptide-peptidoglycan, polysaccharide, or protein bonds. The glycan chain in *E. coli* and Gram-negative bacteria tend to be straight and surround the cell. *Escherichia coli* contains 106 repeats of tetrapeptide-disaccharide units or sufficient for two or three layers of peptidoglycan. Meanwhile, a Gram-positive cell can contain 20 times the peptidoglycan, sufficient for 40 layers or more (Brock et al. 2006).

The bacteriostatic/bacteriocidal activity in Figures 7 and 8 showed 500 mg/mL and 250 mg/mL of ethanol extract against *E. coli* bacteria, 1000 mg/mL and 500 mg/mL of ethanol extract against *B. subtilis* were bacteriostatic. It can be seen based on the color produced that does not match the control media, where the color of each extract is cloudy while the color of the controlled media is clear. The cloudy color indicates that there is still bacterial growth. It suggests that the concentration inhibits the growth of *E. coli* bacteria and *B. subtilis*.



Figure 7. Bacteriostatic/bacteriocidal activity at concentrations of 500 mg/mL and 250 mg/mL ethanolic extract of *R. tuberosa* leaves against *E. coli* bacteria

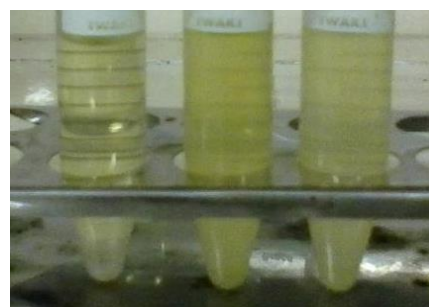


Figure 8. Bacteriostatic/bacteriocidal activity at concentrations of 1000 mg/mL and 500 mg/mL ethanol extract against *B. subtilis* bacteria

Phytochemical screening of *R. tuberosa* leaf extract with ethanol and n-hexane

Phytochemical screening determined the presence of secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, saponins, and glycosides in the test extract. The obtained ethanol and n-hexane extracts were then tested for phytochemical screening using thin TLC plates (Wagner and Zgainski 1984). Phytochemical screening of ethanol and n-hexane extracts can be seen in Figure 9 A-F.

In the screening of alkaloids, the principle is a precipitation reaction due to ligand replacement. The nitrogen atom, which has a lone pair of electrons on the alkaloids, can replace the iodine ion in the Dragendorff reagent (Marliana et al. 2005). Alkaloids can be found in various parts of plants, but the levels of alkaloids in plant tissues are often less than 1% (Kristanti et al. 2008).

Flavonoids are part of phenolic compounds. Flavonoids have ortho-positioned hydroxy groups that produce yellow fluorescence at UV 366 when reacted with cytochrome. Flavonoids have a type that exists in the free form (aglycone) or is bound as glycosides. Polymethoxy aglycones are non-polar, polyhydroxy aglycones are semi-polar, while flavonoid glycosides are polar because they contain several hydroxyl groups and sugars (Harborne 2006). Therefore, the flavonoid group can be attracted to the polar solvent of ethanol.

The main properties of plant tannins depend on the phenolic group -OH contained in the tannins. The color change indicates tannins after being sprayed with FeCl₃, which can react with one of the hydroxyl groups in the tannin compound. Spraying FeCl₃ produces a green-black color that shows condensed tannins (Harborne 2006).

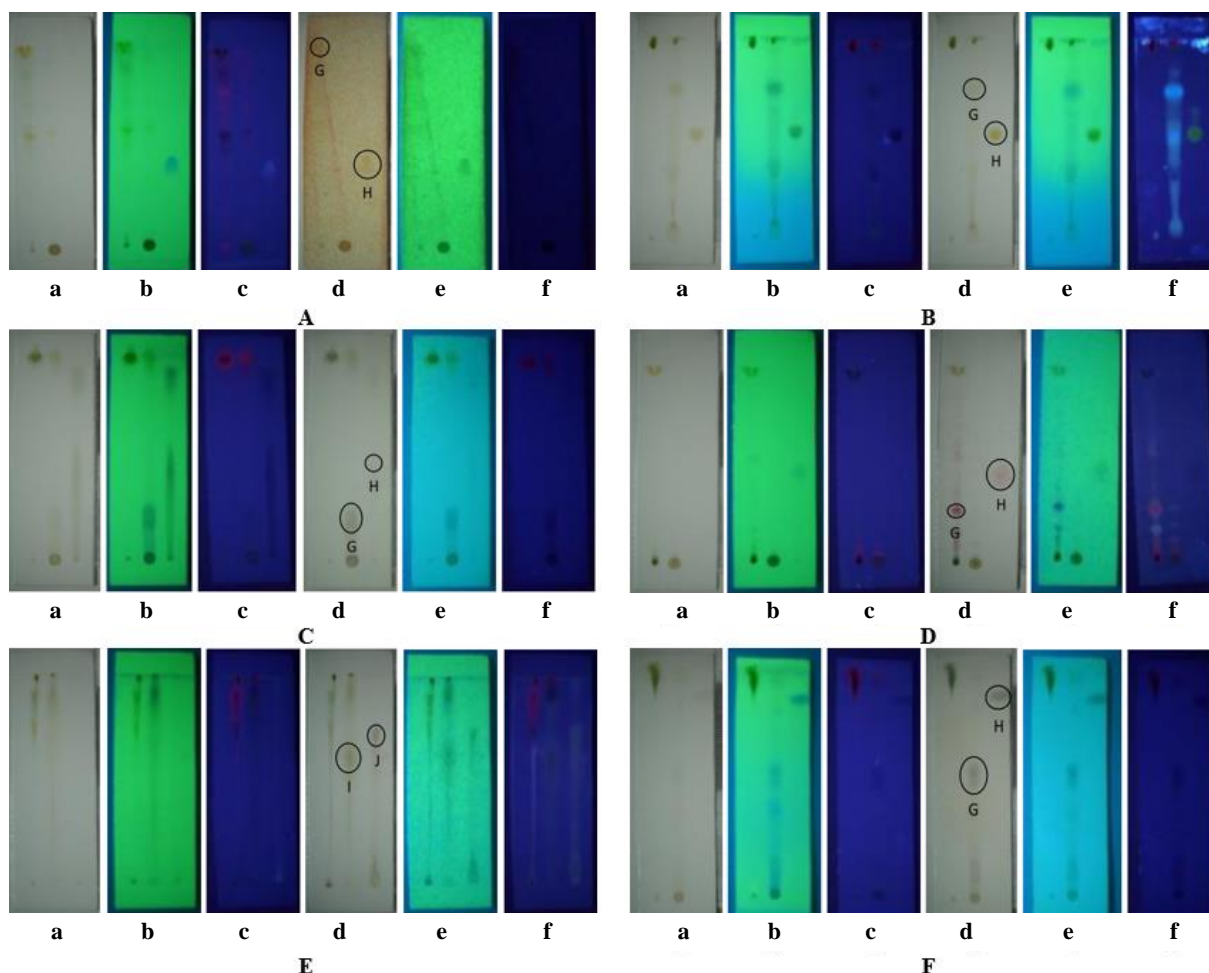


Figure 9. TLC chromatogram on *Ruellia tuberosa* leaves extract. A. The formation of orange spots indicated phytochemical screening of alkaloid compounds after spraying with Dragendorff in visible light; B. Phytochemical screening of flavonoid compounds was indicated by the formation of yellow spots after being sprayed with Sitroborat in visible light; C. Phytochemical screening of tannin compounds was indicated by the formation of dark green-black spots after being sprayed with FeCl₃ in visible light; D. Phytochemical screening of terpenoid compounds was indicated by the formation of purplish-red spots after being sprayed with sulfuric acid anisaldehyde in visible light; E. Phytochemical screening of saponin compounds was indicated by the formation of blackish-brown spots after spraying with Liebermann Bouchard in visible light; F. Phytochemical screening of glycoside compounds was indicated by the formation of dark green-black spots after being sprayed with FeCl₃ in visible light. Note: a. Before spraying on visible light; b. Before spraying on UV light 254 nm; c. Before spraying on UV light 366 nm; d. After being sprayed on visible light; e. After being sprayed on UV light at 254 nm; f. After being sprayed on UV light at 366 nm; G. Rf of ethanolic extract of *R. tuberosa* leaves (A. 0.94, B. 0.75, C. 0.21, D. 0.26, E. 0.6, F. 0.56); H. Rf for comparison (A. quinine 0.42, B. rutin 0.54, C. tannins 0.46, D. tymol 0.42, E. saponins 0.71, F. gallic acid 0.92); I. Rf of saponins ethanol extract of *R. tuberosa* leaves (0.6), J. Rf of saponins as comparison (0.71)

Terpenoids are components commonly found in plants. Most terpenoids contain multiples of five carbon atoms. Terpenoids have a carbon skeleton consisting of two or more C₅ units called isoprene (Nagegowda 2010). In the terpenoid test, the compound analysis was based on the ability of these compounds to form colors with concentrated H₂SO₄. The results obtained showed positive results with the formation of purplish-red spots.

The biosynthesis of terpenoids in plants follows the acetic-mevalonic acid pathway. Acetic acid is activated by coenzyme A to form acetyl-CoA and carry out a condensation reaction with other acetyl-CoA to develop acetoacetyl-CoA. The formed acetoacetyl CoA also condenses with other acetyl CoA units, resulting in three combined units of acetyl-CoA, which are further protonated to form mevalonic acid. In the presence of pyrophosphate in mevalonic acid, the release of CO₂ components (decarboxylation) and the release of OPP⁻ form isopentenyl pyrophosphate (IPP) with its isomer dimethylallyl pyrophosphate (DMAPP) (Dewick 2009).

Saponins are a form of glycosides from polar sapogenins so that ethanol solvents can attract them. Saponins are found mainly in plants. The name saponins are taken from the genus of a plant, namely *Saponaria*, the root of the Caryophyllaceae family can be made into soap (Kristanti et al. 2008).

Phytochemical screening of the ethanolic extract of *R. tuberosa* leaves containing alkaloids, flavonoids, tannins, saponins, and glycosides. Meanwhile, the n-hexane extract of *R. tuberosa* leaves contains terpenoid compounds. The presence of secondary metabolites in each extract was due to the polarity of each solvent that could attract these compounds. Alkaloid compounds, flavonoids, tannins, saponins, and glycosides can generally be attracted by polar solvents such as ethanol (Ncube et al. 2008), while terpenoids are non-polar compounds, which can generally be attracted to non-polar solvents such as n-hexane (Tiwari et al. 2011).

Based on literature data on phytochemical and biological investigations of the genus *Ruellia*, the compounds known as secondary metabolites of this genus are flavonoids, alkaloids, terpenoids, and glycosides. They have pharmacological activities, such as antibacterial (Samy et al. 2015). Vasantharaj et al. (2013) stated that the phytochemical tests showed the antibacterial activity of the methanolic extract of *R. tuberosa* leaves due to phytochemical compounds such as alkaloids, terpenoids, tannins, glycosides, and saponins found in these plants.

In conclusion, based on the research results that have been carried out, the following conclusions can be drawn: (i) *R. tuberosa* leaf extract with ethanol and n-hexane as solvents have antibacterial activity against *E. coli* and *B. subtilis*. (ii) Minimum inhibitory concentration of *R. tuberosa* leaf extract with ethanol as a solvent against *E. coli* was 500 mg/mL with an inhibition percentage of 99.1%. In comparison, the minimum inhibitory concentration value for *B. subtilis* was 1000 mg/mL with an inhibition percentage of 99%. (iii) Chemical compounds contained in the leaf extract of *R. tuberosa* with ethanol solvent are alkaloids, flavonoids, tannins, saponins, and

glycosides. Meanwhile, the solvent n-hexane contains terpenoid compounds.

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