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Antibacterial activity test on ethanol extract fraction of Kirinyuh (*Chromolaena odorata* L.) leaves for multi-drug resistant organisms bacteria

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ABSTRACT. The resistance of pathogenic bacteria to antibiotics is increasing due to antibiotics with incorrect doses, wrong diagnostics, and the wrong target. Bacteria that have been resistant to several antibiotics are called multi-drug resistant organisms (MDRO) bacteria. Bacterial resistance to some antibiotics requires alternative herbal treatments, one of which is the *Chromolaena odorata* L. Research must therefore be conducted on the antibacterial activity of the ethanol extract fraction of *C. odorata* L. leaves for MDRO bacteria, such as *Staphylococcus lugdunensis* methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* extended-spectrum beta-lactamase (ESBL), and *Klebsiella pneumoniae* ESBL. This study aims to determine the antibacterial activity of the ethanol extract of Kirinyuh leaves (*C. odorata* L.) and the antibacterial activity of the fractionation against MDRO bacteria. Test results of kirinyuh leaf ethanol extract for *S. lugdunensis* MRSA, *P. aeruginosa* ESBL, and *K. pneumoniae* ESBL bacteria each resulted in an inhibition zone with an average diameter of 11.6 mm (strong), 11.5 mm (strong), and 11.13 mm (strong), respectively. Testing the antibacterial activity of the ethanol fraction against MDRO bacteria can show antibacterial activity against all tested bacteria, namely Fraction 5. The results of the antibacterial activity of fraction 5 against *K. pneumoniae* ESBL, *P. aeruginosa* ESBL, and *S. lugdunensis* MRSA bacteria with the formation of inhibition zones formed of 10.2 mm (strong), 8.8 mm (moderate), and 7.9 mm (moderate), respectively. The results of thin-layer chromatography showed that the secondary metabolites contained in the fifth fraction were terpenoids, steroids, and flavonoids.

Keywords: ESBL; fraction 5; inhibition zone; multi-drug resistant organisms; secondary metabolites

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INTRODUCTION

The cases of bacterial resistance to antibiotics are a serious problem in medicine (Nathan & Cars, 2014; Setiawati, 2015). The study results revealed that around 40-62% of antibiotics are used inappropriately for several diseases that do not require antibiotics (Yarza *et al*., 2015). Irrational use of antibiotics will have negative effects, such as glycopeptide immunity of microorganisms to some antibiotics, increased drug side effects, and even fatality (Binda *et al*., 2014; Pratiwi, 2017; Rather *et al*., 2017). According to The Centers for Disease Control and Prevention, USA (CDC, 2019), a total of 35000 patients died each year due to resistant bacterial infections. Bacteria that have been resistant to several antibiotics are called multi-drug resistant organisms (MDRO) bacteria.

Rahmantika *et al.* (2016) found MDRO bacteria-infected patients in the pediatric intensive care unit, including *Pseudomonas* sp., *Klebsiella* sp., *Serratia* sp., *Enterobacter* sp., *Acinetobacter* sp., *Staphylococcus aureus*, *Escherichia coli* sp., *Moraxella* sp., *Yersinia* sp. At the same time, *Edwardsiella* sp., Estiningsih *et al.* (2016) found that infected patients in the neonatal intensive care unit. MDRO bacteria have also been found in patients with pneumonia. The most prevalent pneumonia-causing bacteria are *Klebsiella pneumoniae* (46%), *Streptococcus* sp. (24%), *Klebsiella oxytoca* (16%), and *Staphylococcus aureus* (12%) (Alfarizi, 2017). A study by Amelinda *et al.* (2014) shows that the bacteria most commonly cause pneumonia-like infections are *Klebsiella pneumoniae* with 328 isolates (53.16%), *Streptococcus α-hemolyticus* with 104 isolates (16.86%), and *Pseudomonas* sp.

with 54 isolates (8.75%). Pneumonia cases also occurred repeatedly in a 69 year-old man, caused by the organism *Staphylococcus lugdunensis* (Mbaebie *et al*., 2018).

Bacterial resistance to some antibiotics requires other alternative treatments derived from plants. One type of plant that has such properties is "komba-komba" (vernacular name) or Kirinyuh (*Chromolaena odorata* L.) (Syahruramadhan *et al*., 2016). *C. odorata* L. has been reported to show antibacterial, antiplasmodic, antiprotozoal, antitrypanosomal, antifungal, antihypertensive, anti-inflammatory, astringent, antimalarial, antihypertensive, diuretic, hepatotropic (Hanh *et al*., 2011; Priono *et al*., 2016), immunomodulatory, and anticancer effects (Torrenegra & Rodríguez 2011; Harun *et al*., 2012; Subramoniam *et al*., 2012; Kouamé *et al.*, 2013; Vijayaraghavan *et al*., 2017).

Krinyuh leaves contain metabolite compounds such as alkaloids, flavonoids, tannins (Damayanti *et al*., 2013), glycosides, saponins, and steroids/triterpenoids (Marianne *et al*., 2014; Hidayatullah, 2018). Previous studies regarding the antibacterial activity of *C. odorata* extract were limited only to clinical diarrhea strains such as *Bacillus cereus, Escherichia coli, Klebsiella oxytoca, Salmonella enterica, Salmonella typhimurium, Shigella sonnei*, and *Vibrio cholera*, and skin infections due to bacteria such as *Staphylococcus epidermidis* (Naidoo *et al.*, 2011; Atindehou *et al*., 2013; Eze *et al*., 2013). Several reports have examined the effects of *C. odorata* extract on bacterial strains of skin infections in humans (Hanphakphoom *et al*., 2016). A particular study (Alabi *et al.*, 2019) have tested the *C. odorata* L. leaf extract against MDRO bacteria isolated from wounds, and it has been reported that crude and aqueous ethanol extract of *C. odorata* leaves has anti-methicillin-resistant *Staphylococcus aureus* (MRSA) properties (Okwu *et al.*, 2014).

This study was conducted on MDRO bacteria that cause pneumoniae, including *Staphylococcus lugdunensis* MRSA, *Klebsiella pneumoniae* ESBL (extended-spectrum beta-lactamase), and *Pseudomonas aeruginosa* ESBL using ethanol extract from *C. odorata* L. leaves and ethanol chloroform fractionation. This study aims to determine the antibacterial activity of the ethanol extract of *C. odorata* L. leaves and the fractionation against MDRO bacteria. This research is expected to provide important information regarding the potential of local plants that can be utilized as ethnopharmacology.

MATERIALS AND METHODS

The sample used in this study are kirinyuh leaves (*Chromolaena odorata* L.) and three multi-drug resistant-organisms (MDRO) bacterial isolates: *Staphylococcus lugdunensis* MRSA, *Klebsiella pneumoniae* ESBL and *Pseudomonas aeruginosa* ESBL obtained from the Laboratory of Microbiology, Department of Clinical Pathology, University Hospital of North Sumatra, Medan.

Leaf sample preparation. Fresh *C. odorata* L. leaves were obtained from Jl. Tongkoh, Dolat Rakyat, Berastagi District, Karo Regency, North Sumatera Province. Leaf samples that had been picked were fresh green, not moldy, not rotten with leaf sequence number 3 from leaf top to sequence 7. The leaf number 3 from the shoot had undergone physiological maturation and had a maximum secondary metabolite content (Manguntungi *et al*., 2016). Leaves were cleaned with running water, then dried at room temperature protected from sunlight for ± five days. The dried samples were blended and filtered using a 60 mesh sieve.

Leaf extraction. A total of 250 gr of *C. odorata* L. leaf powder was macerated with 1000 mL 96% ethanol in a vessel for five days, then filtered with Whatman No. 1 filter paper. The waste was then macerated again with the same solvent and ratio for three times of repetitions. The macerates are put together and evaporated using a vacuum rotary evaporator until it becomes a thick extract. The extract is stored in a refrigerator at 40°C (Okigbo *et al*., 2005), with the following immersion calculations:

$$\text{Immersion} = \frac{\text{weight of the viscous extract obtained}}{\text{weight of powder simplicia}} \text{ x } 100\ \%$$

Preparation of bacteria. MDR bacteria rejuvenation is performed by scratching bacterial colonies on Nutrient Agar (NA) media using a loop needle. Bacterial cultures were stored in an incubator for 24 h at 37°C. After incubation, one loop of the regenerated bacterial culture is taken and put into a tube containing a 0.9% NaCl solution (Kursia *et al*., 2016).

The antibacterial activity of the ethanol extract of leaves. This test was carried out using the Kirby-Bauer method. Disc paper that has been dripped with ethanol extract of *C. odorata* L. leaves as much as 20 µL was placed on the surface of Mueller Hinton Agar (MHA) media that had been inoculated with MDR bacteria. Chloramphenicol 50% was used as positive control and ethanol 96% as a negative control. Petri dishes were then incubated for 24 hours at 37ºC (Kursia *et al*., 2016).

Column chromatography. Ethanol viscous extract fractionation used chloroform and ethanol 96% solvents. A little cotton was put at the bottom of the column, then 20 gr of absorbed silica gel was added. The separation of the fractions will occur by adding the solvent as the mobile phase. Two solvents were added, namely chloroform and ethanol, with the following ratios: 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9. It was carried out to obtain the best solvent ratio for separating the compounds (Ritna *et al*., 2016). The fractions were then collected in separate vials and stored for further analysis.

Test of antibacterial properties of the fractionation result. The test for the antibacterial properties of the ethanol extract fraction of *C. odorata* L. leaves underwent the same procedure as the antibacterial activity test of the ethanol extract of *C. odorata* L. leaves. Chloramphenicol was used as a positive control and 96% ethanol as a negative control. Clear zone measurements were taken after 24 h. Fractions with potential antibacterial properties were put under a thin layer chromatography test.

Thin layer chromatography. The TLC plate used is 1 x 10 cm, then it was marked with the upper and lower borders as high as 1 cm. The fraction which has an inhibitory power is placed on the TLC plate using a capillary pipette. The TLC plates that had been dripped then eluted with chloroform:ethanol (6:4, 5:5, 4:6,) as eluent. After the eluent propagation reached the specified height, the TLC plate was removed, dried, and staining was observed using 366 nm UV light (Alegantina & Isnawati, 2010). The Rf value is calculated from each stain using the following formula:

$$Rf = \frac{a}{b}$$

Notes:
a= distance of the spot's center point from the starting point
b= eluent displacement distance

Identification of stains was carried out by spraying ammonia vapor reagent to identify flavonoids that would produce blackish-brown stains (Ramadhani *et al*., 2017). Dragendorff's reagent for the alkaloid test would produce brown and orange stains. In contrast, the Lieberman Buchard's reagent for steroid testing would produce green-blue and red or purple stains on terpenoid compounds (Ance *et al*., 2018).

Data analysis. The data obtained from the study were in the form of inhibition zone results from the antibacterial activity test of ethanol extract and each fraction with a clear zone diameter, as well as the content of secondary metabolites from the color of the stains formed during the thin layer chromatography test. Data is presented in tabular form and interpreted according to the results obtained.

RESULTS AND DISCUSSION

Antibacterial properties test of Kirinyuh (*Chromolaena odorata* L.) leaf crude extract against MDR bacteria. Based on observations, the ethanol extract of *C. odorata* L. leaves had antibacterial properties against *Staphylococcus lugdunensis* MRSA, *Klebsiella pneumoniae* ESBL, and *Pseudomonas aeruginosa* ESBL. The thick evaporated extract was brownish-black and weighed about 27.26 gr, and had a yield value of 9.08%. The results of the observations can be seen in Table 1.

Table 1. The results of antibacterial properties test of *C. odorata* L. leaf ethanol extract

Bacteria	Tx	The avg diameter of inhibition zone (mm)	Potency
Staphylococcus lugdunensis MRSA	EEDK	11.6	strong
	K^+	31.2	very strong
	K^-	-	-
Pseudomonas aeruginosa ESBL	EEDK	11.5	strong
	K^+	37.73	very strong
	K^-	-	-
Klebsiella pneumoniae ESBL	EEDK	11.13	strong
	K^+	34.16	very strong
	K^-		-

Note: Tx= treatment; EEDK= ethanol extract of kirinyuh leaves; positive control (K+)= chloramphenicol antibiotic; negative control (K-)= ethanol 96%

It was provided that the ethanol extract of *C. odorata* L. leaves had antibacterial properties against *S.lugdunensis* MRSA, *P. aeruginosa* ESBL, and *K. pneumoniae* ESBL, as indicated by the formation of each clear zone starting from the largest with an average diameter of 11.6 mm (strong); 11.5 mm (strong); and 11.13 mm (strong). Based on these data, the ethanol extract of *C. odorata* L. leaves has the potential to inhibit growth in gram-positive and gram-negative bacteria. These results probably indicate the presence of a bioactive component with broad-spectrum antibacterial properties (Alabi *et al*., 2019). According to Hanphakphoom *et al*. (2016), ethanol solvent on *C. odorata* L. leaves showed good antimicrobial properties in the antibacterial activity test. Hence the ethanol extract of *C. odorata* L. leaves contains secondary metabolites of phenols, saponins, tannins, glycosides, steroids, terpenoids, and flavonoids (Alabi *et al.*, 2019), which synergize with each other to inhibit bacterial growth.

The antibacterial activity of the ethanol extract of *C. odorata* L. leaves inhibited the growth of gram-positive bacteria was greater than gram-negative bacteria. The gram-positive bacteria have cell walls with larger portion of peptidoglycan, fewer lipids and highly abundant of wall teichoic acids (Reith & Mayer, 2011; Brown *et al*., 2013). The content of polar secondary metabolites such as flavonoids and tannins is easier to penetrate the polar peptidoglycan layer than the non-polar lipid layer. Furthermore, cell walls that are most easily denatured were the cell wall composed of polysaccharides, compared to those composed of phospholipids. Thus, the inhibitory power of *S. lugdunensis* MRSA is greater than that of *K. pneumoniae* ESBL and *P. aeruginosa* ESBL.

Antibacterial properties of fractionation results against MDRO Bacteria. The viscous extract was fractionated using column chromatography with 96% chloroform and ethanol as solvent. According to Alabi *et al*. (2019), solvent mixtures with variable polarity in different ratios can be used to separate pure compounds from plant extracts. Fractionation was carried out using the mobile phase from a less polar solvent to a more polar solvent. The results of the fractionation with chloroform and ethanol 96% solvents can be seen in Table 2.

Table 2. Fractionation results of ethanol extract with chloroform and ethanol 96% solvent

Fraction	Rasio	Total	Color
chloroform:etanol (F1)	9:1	7 ml	Pale stray yellow
chloroform:etanol (F2)	8:2	7 ml	Transparent yellow
chloroform:etanol (F3)	7:3	8 ml	Dark yellow
chloroform:etanol (F4)	6:4	4 ml	Brown
chloroform:etanol (F5)	5:5	7.5 ml	Dark green
chloroform:etanol (F6)	4:6	7.5 ml	Dark green
chloroform:etanol (F7)	3:7	4.8 ml	Dark green
chloroform:etanol (F8)	2:8	5 ml	Brown
chloroform:etanol (F9)	1:9	8 ml	Honey

Fractionation results showed various shades within the yellowish spectrum, including pale stray yellow, transparent yellow, dark yellow, brown, dark green, brown, and honey. The color difference is due to the slow descent of the extract into fractions. In a study by Alabi *et al.* (2019), extract fractionation and purification of bioactive components can provide better antibacterial activity than pure extracts. The nine fractions were subjected to

tests for antibacterial activity against *S. lugdunensis* MRSA, *K. pneumoniae* ESBL, and *P. aeruginosa* ESBL. Antibacterial activity testing was conducted to determine which fraction could inhibit the growth of the tested bacteria. The results of the antibacterial properties test of the fraction against MDRO bacteria can be seen in Table 3.

Table 3. Fractionation results of ethanol extract with chloroform and ethanol 96% solvent

Bacteria	Tx	The avg diameter of inhibition zone	Potency
Staphylococcus lugdunensis MRSA	F1	-	-
	F2	-	-
	F3	-	-
	F4	-	-
	F5	7.9	moderate
	F6	-	-
	F7	-	-
	F8	-	-
	F9	-	-
	K^+	28.4	very strong
	K^-	-	-
Pseudomonas aeruginosa ESBL	F1	-	-
	F2	-	-
	F3	-	-
	F4	-	-
	F5	8.8	moderate
	F6	-	-
	F7	-	-
	F8	-	-
	F9	-	-
	K^+	29.6	very strong
	K^-	-	-
Klebsiella pneumoniae ESBL	F1	-	-
	F2	-	-
	F3	-	-
	F4	-	-
	F5	10.2	strong
	F6	-	-
	F7	-	-
	F8	-	-
	F9	-	-
	K^+	38	very strong
	K^-	-	-

There was a significant difference between *P. aeruginosa* ESBL and *K. pneumoniae* ESBL. As a gram-negative bacterium, *P. aeruginosa* ESBL has a fairly dense and compact peptidoglycan wall and the existence of an efflux-pump mechanism, which is a mechanism for removing compounds that are not needed in the process of bacterial cellular biotransformation through the secretion system. This data was obtained from the North Sumatra University Hospital data related to bacteria's ability to antibiotics from ID ES01 patients with bionumber 0003453103500010 and ID DS11 bionumber 6607734753565210 as a sensitivity test to determine that these bacteria are MDRO bacteria. Based on the sensitivity test, the *P. aeruginosa* ESBL bacteria were resistant to nine types of antibiotics, namely piperacillin/tazobactam, cefazolin, ceftazidime, cefepime, aztreonam, meropenem, gentamicin, ciprofloxacin, and nitrofurantoin. In contrast, the *K. pneumoniae* ESBL bacteria was only resistant to five antibiotics, including ampicillin, ampicillin/sulbactam, ceftriaxone, aztreonam, and nitrofurantoin. The inhibition of bacteria to be greater against the *K. pneumoniae* bacteria. The *S. lugdunensis* MRSA bacteria are also more pathogenic hence resistant to nine types of antibiotics, namely benzylpenicillin, oxacillin, gentamicin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin, and tetracycline.

Results of secondary metabolite thin layer chromatography. The fraction which has antibacterial properties and the inhibition of the clear zone were further tested by thin layer chromatography. This test is carried out to determine the secondary metabolite content in the fraction. The results of thin layer chromatography test for fraction 5 can be seen in Table 4.

Table 4. Results of TLC test on fraction 5 ethanol extract of *C. odorata* L. leaves.

Ratio eluent chloroform: ethanol	Stain	Ch.	Rf value	Content of secondary metabolite
6:4	1	K1	0.9	Terpenoid
5:5	1	K2a	0.7	Flavonoid
	2	K2b	0.8	Steroid
4:6	1	K3a	0.63	Terpenoid
	2	K3b	0.76	Terpenoid
	3	K3c	0.85	Flavonoid

Notes: Ch= Chromatogram; K1= Chromatogram 1; K2a= Chromatogram 2 stain 1; K2b= Chromatogram 2 stain 2; K3a= Chromatogram 3 stain a; K3b= Chromatogram 3 stain 2; K3a= Chromatogram 3 stain 3.

Based on the study results, each eluent comparison produced a varying number of stains, causing the Rf value to vary. The difference in Rf value reflects the polarity, where the content of secondary metabolite compounds with high Rf values in the solvent system has a low polarity, while the lower Rf value has high polarity (Okwu *et al*., 2014). K1, K3a, and K3b produced Rf values of 0.9 cm, 0.63 cm, and 0.76 cm, respectively, which were thought to have terpenoid secondary metabolites. Based on Jangnga *et al.* (2018), terpenoid compounds were characterized by Rf values of 0.92 cm, 0.75 cm, 0.63 cm, and 0.36 cm. K2a and K3c each produced Rf values of 0.7 cm and Rf 0.85 cm, which were thought to contain secondary flavonoid metabolites. According to a study conducted by Rohmah *et al.* (2019), the resulting Rf values for flavonoid compounds were 0.88 cm and 0.83 cm, while Yuhernita & Juniarti (2011) stated that the Rf value of 0.707 was a flavonoid with a greenish-brown color.

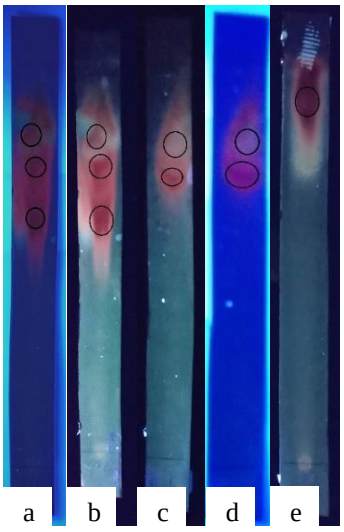

Fig. 1. The appearance of stains on UV 366 light with different eluent ratios after spraying the reagent: a eluent 4:6 after spraying with ammonia; b. eluent 4:6 after spraying with Lieberman; c. eluent 5:5 after spraying with ammonia; d. eluent 5:5 after spraying with Lieberman bourchad; e. eluent 6:4 after being sprayed with Lieberman bourchad.

Then, K2b produced an Rf value of 0.8 cm, which was thought to contain secondary metabolites of steroids. In line with Hidayah *et al.* (2016), the stain with Rf 0.8 is light blue, indicating a steroid compound. Based on these results, it is suspected that secondary metabolite compounds can inhibit bacterial growth, namely steroids, terpenoids, and flavonoids. As reported by Okwu *et al*. (2014), Rf values ranging from 0.3 to 0.9 indicate the presence of terpenes, phenolic acids, and flavonoids. Furthermore, the stained chromatogram was sprayed with reagents to estimate the content of secondary metabolites exposed to UV lamps, as shown in Fig. 1.

Secondary metabolites in fraction 5 contain secondary metabolites of steroids, terpenoids, and flavonoids. Steroids can penetrate the relatively thick cell walls of gram-negative bacteria easily due to their fat-solubility. Thus, fraction 5 has antibacterial potential with a greater clear zone against gram-negative bacteria. The mechanism of action of steroids as antibacterial can damage the lipid membrane so that the liposomes leak. The content of secondary metabolites of steroids/terpenoids has antibacterial properties by inhibiting growth or killing bacteria by interfering with the process of cell wall formation, where the cell wall is formed but imperfectly (Patra & Mohanta, 2014). While, flavonoids can reduce the fluidity of bacterial cell membranes directly related to cytoplasmic membrane damage or indirect damage through autolysis cascade or weakening of the cell walls, resulting in osmotic lysis (Wu *et al*., 2013; Matijašević *et al*., 2016).

CONCLUSION

The ethanol extract from *Chromolaena. odorata* L. leaves has antibacterial properties against bacteria *Staphylococcus lugdunensis* MRSA, *Pseudomonas aeruginosa* ESBL, and *Klebsiella pneumoniae* ESBL, indicated by the formation of clear zones of 11.6 mm (strong), 11.5 mm (strong), and 11.13 mm (strong) respectively. In addition, fraction 5 of ethanol extract showed positive results by producing a clear zone that inhibits *S. lugdunensis* MRSA, *P. aeruginosa* ESBL, and *K. pneumoniae* ESBL bacteria with an average diameter of 7.9 mm (moderate), 8.8 mm (medium), and 10.2 mm (strong), respectively. Secondary metabolite compounds contained in fraction 5 of ethanol extract are flavonoids, steroids, and terpenoids.

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