Zainab 2

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Identification of active compound and antibacterial activity against gram-positive and gram-negative bacteria of *Chromolaena odorata* leaf extract

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ABSTRACT:

Antibacterial from natural ingredients, such as medical plants can be used as an alternative medicine. The aims of this study were to identify active compounds and antibacterial activity from the leaf extract of *Chromolaena odorata*, especially to against gram-positive bacteria and gram-negative bacteria. The methods of this study were making simplicia powder, extracting the leaves using ethanol, phytochemical screening using the tube method with specific reagents. Fractionated with chloroform and ethyl acetate and analyzed by Gas Chromatography–Mass Spectrometry (GC-MS) to identify the active compounds. Antibacterial activity used diffusion method. We also determined the minimum inhibitory concentration (MIC) and minimum kill concentration (MKC). The results of the chloroform fraction showed 14-Methyl-Pentadecanoic Acid; 9-Octadecanoad; 2(2-(2,5-Ditiacyclopentyl)-N-Propyl-3 Isopropanol; Bis (dichloromethyl)-ether. The results on the ethyl acetate fraction were 4-oxopentanoid acid or Levulinic acid; 4,4- dieutero-heptan-1-ol; 1,2,2-trichloro-1,1-difluoromethane; and 1-ethyl-2-methyl-3-oxoheksanoic acid. In addition, inhibition zone leaf extract 10% w/v against *S. epidermidis* 18.44 mm; *S. mutans* 19.32 mm; *S. aureus* 19.50 mm; *P. aeruginosa* 12.70 mm; *E. coli* 0.00 mm; and *S. thypi* 0.00 mm. MIC of *S.aureus*, *S.mutans*, *S epidermidis*, and *P.aeruginosa* were 6250 ppm, 6250 ppm, 1562.5 ppm, and 9375 ppm, respectively. MKC of *S.aureus*, *S mutans*, *S.epidermidis*, and *P aeruginosa* were 6250 ppm, 6250 ppm, 1562.5 ppm, and 12500 ppm, respectively. The conclusions of this study were the leaf extract of *Chromolaena odorata* contained terpenoid, phenolic and flavonoid. Antibacterial activity of this extract showed strong activity on *S. aureus*, *S. mutans*, *S. epidermidis*, and *P. aeruginosa* bacteria.

KEYWORDS: Antibacterial, *Chromolaena odorata*, leaf extract, GC-MS, MIC, MKC

INTRODUCTION :

Antibiotic-resistant bacteria are a global problem in the world, including Indonesia. This condition has occurred because of lack of research[1]. It is necessary to explore antibacterial from natural ingredients, such as medical plants[2,3]. Further, it can be used as an alternative medicine due to effectiveness, safety considerations, as well as the cost[4,5].

Indonesia has the second biggest which has medical plants in the world. The natural wealth of Indonesian medicinal plants consists of 25,000 and 30,000 species[6]. Medicinal plants have been used since ancient time and can be used for treatment the disease[7]. One of the plants used for alternative medicine is *Chromolaena odorata*, called kelambu menjangan in Kalimantan island, Indonesia. *Chromolaena odorata* leaf is used to treat skin disease, such as *Propionibacterium acnes*, anti-inflammatory, analgesic, antioxidant, antifungal, as well as antibacterial effects[3]. Previous study mentioned that leaf extract *Chromolaena odorata* has antibacterial activity against *Staphylococcus aureus*[8,9]. However, there is still limited reference in terms of antibacterial activity against the others bacteria.

Based on this background, the aim of this study was to identify active compounds and antibacterial activity from the leaf extract of *Chromolaena odorata*, especially against gram-positive bacteria, *Staphylococcus aureus*, *Streptococcus mutans*, and *Staphylococcus epidermidis* and gram-negative bacteria, *Escherichia coli*, *Pseudomonas euroginosa*, and *Salmonella typii*.

MATERIALS AND METHODS :

This was a true experimental study to identify active compounds and antibacterial activity from the leaf extract of *Chromolaena odorata*.

Instruments

The sample for this study was leaves of *Chromolaena odorata*. The instruments and the materials were used in this study: 96% ethanol Eralika®, quercetin Sigma®, ethanol pro-analytical Merck®, AlCl3 Pudak®, pro-analytical acetic acid Merck®, sterile aqua Eralika®, paper disc steril Sigma®, nutrient agar (NA), sterile cotton swab, sterile paper disc, gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175, *and Staphylococcus epidermidis* ATTC 12228 and also gram negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas auroginosa* ATCC 9027, and *Salmonella typhi* ATCC 786, dimethyl sulfoxide (DMSO) Sigma®, chloramphenicol Merck®nutricat broth (NB) Sigma®, Mayer's reagent Eralika®, foam, $FeCl_3$, gelatin, alkali, Lieberman-Buchard test Eralika®, Salkowski's test Eralika®, glassware, glass vessels, water baths, ovens, lamina air flow, sterilizers, rotary tools.

Plant material

The leaves of the *Chromolaena odorata* were taken according to the specified criteria: fresh, undamaged and mature. The collection was carried out on the Martapura riverbank in June 2021 for two days Macroscopic test of *Chromolaena odorata* leaves was conducted at the Basic Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University. *Chromolaena Odorata* leaves has triangular shape, three bones, opposite branches, and the smell is distinctive and more likely pungent odor. The stems of *Chromolaena odorata* are covered with fine hairs [10].

Extractions process

Chromolaena Odorata were sorted by wet sorting, drained, and baked in an oven at 55°C. After drying, we conducted dry sorting and stored it in dry form. One kg of dry leaf powder was extracted using 96% ethanol in a ratio of 1: 10. Extraction was conducted for 3 x 24 hours and we changed the solvent every 1 x 24 hours. We filtered the extraction and obtained liquid extract. The liquid extract was evaporated using a rotary evaporator until shrinking to 1/10.

Phytochemical screening

Phytochemical screening was dissolved into the exttact with 1% concentration. From this solution, 1 ml was taken and then mixed with each reagent, five drops of alkaloids using dragendroff reagent, saponins aquadest, phenolics using iron chloride, tannins using gelatine 1 %, flavonoids using sodium hydroxide, steroids and terpenoids using Lieberman -Buchard. Then this extract was fractionated using chloroform[11] and ethyl acetate solvents[12]. The fraction was evaporated using a rotary evaporator and concentrated using a water bath to obtain a thick extract.

Gas Chromatography – Mass Spectroscopy

Identification all fractions used Gas Chromatography – Mass Spectroscopy (GC-MS) analysis. I μl of the chloroform fraction was used in GC-MS for the analysis of different compounds. Instruments and chromatographic conditions carried out on the GC-MS HP 6890 system. 1 μl of sample was injected into the GCMS. We used column with capillary model number agilent 19091S-433 HP-5MS 5% Phenyl Methyl Siloxane, length 30 m, diameter 250 μm, and thick 0.25 μm. The oven temperature was between 100-220°C. The temperature rise rate was 15°C/mln and the flow rate is 1.0 ml/min. The carrier gas was helium with a pressure of 10.5 psi, a total rate of 140 ml/min and a split ratio of 1:50. The eluted component was detected in the mass detector. The known spectrum of compound components was stored in the NIST library and the compound name and molecular weight also determined. GC-MS was a method for separating metabolic compounds as well as breaking down using high heating; however, the limitation of this method was it is only detected heat-resistant compound[13 15].

Inhibition Zone Diameter

The diameter of inhibition zone of the extract was tested by the diffusion method using Kirby Bauer Disc Diffusion method. Blank paper discs were dipped into each extract and control group. Then we attached it into the surface of the media that had been smeared with bacteria. We incubated it at 37°C for 24 hours. The diameter of the clear zone formed was measured using ruler. The diameter of the inhibition zone can be calculated by the formula[16]:

$$\frac{d1+d2}{2} - x$$

d1 = vertical diameter of the clear zone on the media.
d2 = horizontal diameter of the clear zone on the media.
X = paper disc diameter (6 mm).

The minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) test was carried out using the microdilution method. Bacterial concentration was adjusted using 0.5 McFarland (105 colonies/mL). 0.1 L of bacterial suspension was put into a polypropylene PP centrifuge tube that contained 0.7 L of NB then we added 0.2 L of sample. This treatment was conducted on all tubes, with different concentrations[17]. Then we incubated it at 37°C for 24 hours. After incubation, the tube containing the bacteria and the extract sample was smeared on the surface of the NA medium in five petri dishes, then we also incubated it at 37°C for 24 hours. The positive control was mixture of bacterial suspension with NB, while the negative control was a mixture of extracts and fractions with NB. A positive result was if there was bacterial growth on the agar medium and a negative results if there was no bacterial growth on the agar medium. The results of the minimum inhibitory concentration were taken from the lowest concentration or no bacterial growth[18].

The minimum killing concentration (MKC)

The minimum killing concentration (MKC) test was carried out using streak plate method. The NA media was put into a petri dish and allowed to solidify, then these samples were taken onto the surface of the NA media using a cotton swab. We incubated it at 37°C for 24 hours. The results with no bacterial growth were considered as MKC. A clear area on a petri dish indicated no bacterial growth[18].

Results and Discussion

Chromolaena odorata was one of medicinal potential plants[19] . In Kalimantan Island, Indonesia, it is known as Kelambu Menjangan and harvested in the Martapura riverbank area. *Chromolaena odorata* can be cultivated in various lands, *Chromolaena odorata* has many benefits for medicine. However, it is rarely used because it is considered as a nuisance plant[20].

Phytochemical Screening

Table 1 showed the metabolite contain of *Chromolaena odorata* leaf extract, such as alkaloids, saponins, phenolics, tannins, flavonoids, steroids and terpenoids. These metabolites function as anti-bacterial. This was similar with previous studies that found the ethanolic extract of *Chromolaena odorata* leaf consists likewise [21,22].

Table 1. Phytochemical screening on *Chromolaena odorata* leaf extract

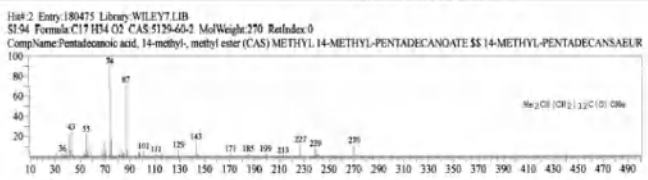
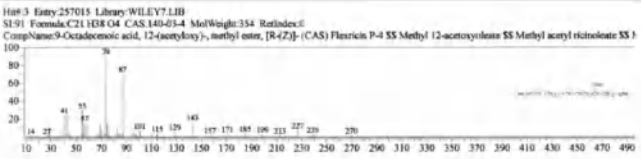
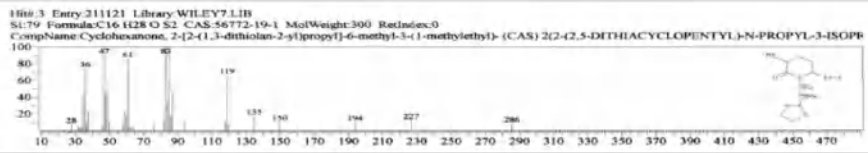
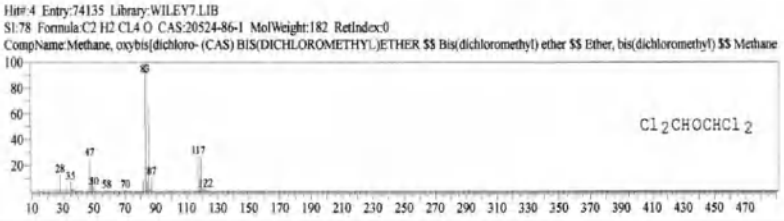
Test	Methods	Description	Results
Alkaloid	Mayer Test	White precipitate	+
Saponin	Foam Test	Foam	+
Fenolik	FeCl3 Test	Bluish black color	+
Tanin	Gelatine Test	White precipitate	+
Flavonoid	Alkali Test	Yellow precipitate	+
Steroid	Lieberman Test	Brown ring precipitate	+
Terpenoid	Salkowski's Test	Golden color	+

GC-MS identification

Chloroform fraction

Table 2 showed that the most predominantly compounds was terpenoid group: 14-methyl-pentadecanoic acid and 9-octadecanoic acid and Bis (dichloromethyl)-ether. In addition, the other compounds that have been identified was the flavonoid group.

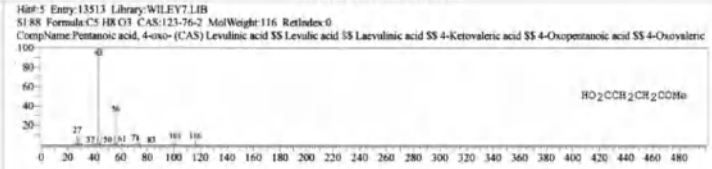
Table 2. Compound identification *Chromolaena odorata* leaf extract based on the chloroform fraction

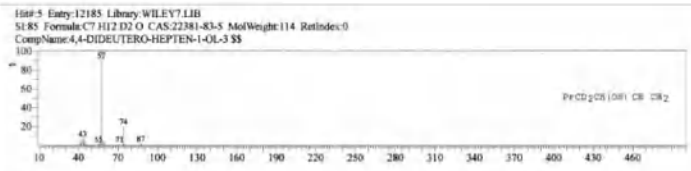
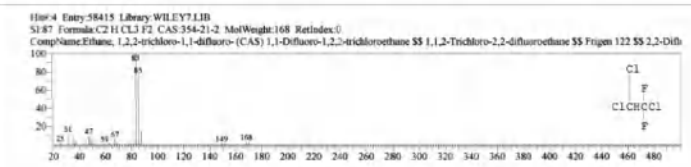
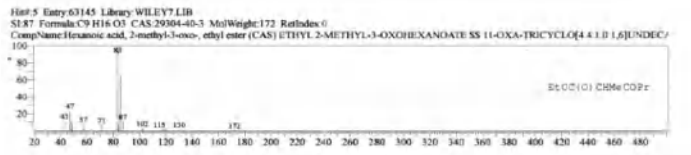
Compound	GC-MS identification
14-methyl-pentadecanoic acid	
Octadec-9-enoic acid	
2(2-(2,5-Dithia Cyclopentyl)-N-Propyl-3-Isopropana	
Bis (dichloromethyl)-ether	

Ethyl Acetate Fraction

Table 3 showed compounds in the terpenoid group: 4-oxopentanoic acid or levulinic acid and 1,2,2-trichloro-1,1-difluoromethane, and another compound was the phenolic group.

Table 3. Compound identification *Chromolaena odorata* leaf extract based on the Ethyl Acetate fraction

Compound	GC-MS identification
4-oxopentanoic acid	

4,4-dideutero-hepten-1-ol	
1,2,2-trichloro-1,1-difluoromethane	
1-etil-2-metil-3-oksoheksan acid	

Based on the results, the most predominant group in *Chromolaena odorata* leaf extract was terpenoid group. Terpenoids were found in roots, stems and bark, flowers and mostly in the leaves. Terpenoids were the largest group of natural products from plants with more than 40,000 structures[23].

Anti-bacterial activity refers to the chemical components, produced by these plants and had function to inhibit the growth of pathogenic bacterial. The anti-bacterial activity of *Chromolaena odorata* was related to the phytochemical content such as phenols, flavonoids, and tannins[24, 25]. Anti-bacterial has function to damage cell walls, plasma membranes, and inhibit protein synthesis; nucleic acid; and the synthesis of important metabolites in bacteria[26].

Diameter Zone of Inhibitory Effect against bacteria

Anti-microbial activity test of 10% ethanol extract showed inhibitory activity against four bacteria, namely *Pseudomonas euroginosa, Staphylococcus aureus, Streptococcus mutans*, and *Staphylococcus epidermidis* with the formation of inhibitory zone around the paper disc. In addition, the positive control only had inhibition on five bacteria because chloramphenicol was not strong enough to inhibit *Pseudomonas euroginosa*.

Table 4. The diameter zone of the inhibitory of gram-positive bacteria

SAMPLE	Inhibitor zone (mm)			Mean (mm)	SD
	1st replication	2nd replication	3rd Replication		
Control positive on *S. epidermidis*	22.18	22.48	22.38	22.35	0.15
Control positive on *S. mutans*	23.17	23.64	23.61	23.47	0.26
Control positive on *S. aureus*	23.7	25.7	13.4	20.93	6.60
Control positive on *P. aeroginosa*	0	0	0	0	0
Control positive on *E. coli*	12.8	30.3	30.3	24.47	10.10
Control positive on *S. thypi*	30.3	30.4	12.6	24.43	10.24
Extract on *S. epidermidis*	18.43	18.44	18.46	18.44	0.01
Extract on *S. mutans*	18.89	19.49	19.57	19.32	0.37
Extract on *S. aureus*	20.4	18.3	19.8	19.50	1.08
Extract on *P. aeruginosa*	12.7	12.2	13.2	12.70	0.50
Extract on *E. coli*	0	0	0	0	0
Extract on *S. thypi*	0	0	0	0	0

Table 4 showed that *Chromolaena odorata* leaf exttact had effect against gram-positive bacteria: *Staphylococcus epidermidis*, *Streptococcus mutans*, and *Siaphylococcus aureus*. *Leaf extract* only had effect against gram-negative bacteria such as *Pseudomonas aeruginosa*. Antibacterial activity on gram-positive bacteria is easier than gram-negative bacteria. Cell wall of gram-positive bacteria has a thick peptidoglycan outer but almost no lipopolysaccharide, so it is easy to penetrate into gram-positive bacteria. While gram-negative bacteria has thin peptidoglycan but it has high lipopolysaccharide (LPS) outer, so it is not easy to penetrate by lipophilic solute[27, 28].

Inhibitor zone on *Chromolaena odorata* means it has antibacterial activity such as terpenoids, phenolics and flavonoids. Terpenoids have capability to cell signaling, metabolism, and biosynthesis[29] as well as to damage porins[30]. Damage in porin will reduce the permeability of the bacterial cell wall and lack of nutrients, so the growth will be inhibited. Phenolics act as anti-oxidants[31] and have capability to inhibit and prevent the growth process of microbial cells. Flavonoids are as antioxidants[32, 33], and can damage permeability bacterial cell walls through the interaction between flavonoids and bacterial DNA. In addition, the hydroxyl group in the sttucture of fiavonoid compounds had toxic effects on bacteria[34].

Range inhibitory diameter zone between 15-20 mm means strong inhibition, 10-14 mm means moderate inhibition and 0-9 mm inhibition zone diameter means weak inhibition[35]. This study showed inhibitory zone of 12.07 mm (moderate inhibition) inhibited *Pseudomonas euroginosa*. In addition inhibitory zone between 15-20 mm (strong inhibition) inhibited all gram-positive bacteria. Based on data, it showed that *Chromolaena odorata* leaf extract can be used as an alternative medicine to treat infectious diseases due to *Pseudomonas euroginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Staphylococcus epidermidis*. This study also in line with previous study that mentioned ethanol extract of the leaves of Chromolaena odorata had strong antibacterial activity against *Streptococcus mutans*, *Staphylococcus epidermidis*, *Staphylococcus aureus*[36].

Table 5. The results of inhibitory test among control gronp and extract

	PCSE	PCSM	PCSA	PCPA	PCEC	PCST	ESE	ESM	ESA	EPA	EEC	EST
PCSE		1.000	1.000	0.000*	1.000	1.000	0.995	0.999	1.000	0.344	0.000*	0.000*
PCSM	1.000		1.000	0.000*	1.000	1.000	0.964	0.991	0.994	0.211	0.000*	0.000*
PCSA	1.000	1.000		0.000*	0.998	0.993	1.000	1.000	1.000	0.565	0.000*	0.000*
PCPA	0.000*	0.000*	0.000*		0.000*	0.000*	0.002*	0.001*	0.001*	0.078	1.000	1.000
PCEC	1.000	1.000	0.998	0.000*		1.000	0.890	0.958	0.967	0.129	0.000*	0.000*
PCST	1.000	1.000	0.998	0.000*	1.000		0.893	0.959	0.968	0.131	0.000*	0.000*
ESE	0.995	0.964	1.000	0.002*	0.890	0.893		1.000	1.000	0.916	0.002*	0.002*
ESM	0.999	0.991	1.000	0.001*	0.958	0.959	1.000		1.000	0.820	0.001*	0.001*
ESA	1.000	0.994	1.000	0.001*	0.967	0.963	1.000	1.000		0.795	0.001*	0.001*
EPA	0.344	0.211	0.565	0.078	0.129	0.131	0.916	0.820	0.795		0.078	0.078
EEC	0.000*	0.000*	0.000*	1.000	0.000*	0.000*	0.002*	0.001*	0.001*	0.078		1.000
EST	0.000*	0.000*	0.000*	1.000	0.000*	0.000*	0.002*	0.001*	0.001*	0.078	1.000	

*significance difference

The statistical tests showed that between the positive control *Staphylococcus epidermidis* (PCSE), *Staphylococcus aureus* (PCSA), *Pseudomonas euroginosa* (PCPA), and extract group of *Streptococcus mutans* (ESM) there was no significant difference between the groups. It means that the extract has similar ability with the positive control (chloramphenicol) to kill these bacteria. The statistical tests showed that comparing the positive control *Escherichia coli* (PCEC) with extracts *Escherichia coli* (EEC) showed significance difference between these two groups. Based on the data, the extract did not have the ability to inhibit *Escherichia coli* as well as *Salmonella typhi* (PCST).

Minimum Inhibitory Concentration (MIC) and Minimum Killing Concentration (MKC)

Tabel 6 showed the MIC and MKC test on the growth of *Staphylococcus aureus*, *Streptococcus mutans*, and *Staphylococcus epidermidis*.

Table 6. Minimum inhibitory concentration (MIC) and minimum killing concentration (MKC)

Minimum inhibitory concentration (MIC)									
Sample	Concentration	*S. aureus*		*S.mutans*		*S.epidermidis*		*P. aureginosa*	
	(%)	R1	R2	R1	R2	R1	R2	R1	R2
Extract	10								
	3	-	-	-	-	-	-	-	-
	2.5	-	-	-	-	-	-	-	-

	1.25	-	-	-	-	-	-	-*	-
	0.625	-*	-*	-*	-*	-	-	+	-*
	0.312	+	+	+	+	-	-	+	+
	0.156	+	+	+	+	-*	-*	+	+
***Minimum Killing Concentration* (MKC)**									
Extract	10	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	2.5	-	-	-	-	-	-	-	-
	1.25	-	-	-	-	-	-	-*	-*
	0.625	-*	-*	-*	-*	-	-	+	+
	0.312	+	+	+	+	-	-	+	+
	0.156	+	+	+	+	-*	-*	+	+

(+) : there was bacterial growth
(-) : There was no bacterial growth
(-*) : MIC or MKC value

The results of MIC and MKC for *Staphylococcus aureus* and *Streptococcus mutans* at a 6250 ppm (0.625% w/v) and *Staphylococcus epidermidis* at 1562.5 ppm (0.156% w/v). Based on the MIC test, the growth of *Pseudomonas euroginosa* at 9375 ppm (0.9375% w/v) and the MBC the growth of *Pseudomonas euroginosa* is at 6250 (0.6250 % w/v), 3125 (0.3125% w/v) and 1562.5 (0.156% w/v). These results indicated that the concentration of the ethanol *Chromolaena odorata* leaf extract has not been able to kill at 12500 (1.25% w/v). Further, there is no bacterial growth for *Pseudomonas euroginosa* at these concentrations.

CONCLUSION

This study showed that the *Chromolaena odorata* leaf extract contain various terpenoid, phenolic and flavonoid compounds. Antibacterial activity of the *Chromolaena odorata* leaf extract showed strong activity against *Staphylococcus aureus*, *Streptococcus mutans*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*.

CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

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