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ISOLATION AND IDENTIFICATION SECONDARY METABOLITES COMPOUND ETHYL ACETATE : n-HEXANE (4 : 6) FRACTION OF GULMA SIAM LEAVES (*Chromolaena odorata* L.)

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Abstract

This study aims to isolate and identify the secondary metabolites compound ethyl acetate : n-hexane (4: 6) fraction from Gulma siam leaves (*Chromolaena odorata* L.), which is derived from Enrekang regency, South Sulawesi, Indonesia. The stages of research include extraction, fractionation, purification and identification. Fractionation is done by flash column chromatography; purification by recrystallization and identification using color test, melting point, solubility, KLT and spectroscopic methods. Obtained compound in the form of white crystal with a melting point about 144-145^{o}C, also react positively with Dragendorff and Wagner reagent that is marked by orange solution and brown precipitants which indicate the possibility of containing secondary metabolites compound that includes in alkaloid class. It is also supported by the presence of N-H absorption around 3329.14 nm; aliphatic C-H around 2916.37 nm; aromatic C-C around 1620.21 nm; C-N around 1357.89 nm and C-O around 1068.50 nm that is obtained on the *IR* spectrum.

Key words : Isolation, Gulma Siam , Identification, alkaloids, IR

INTRODUCTION

Chromolaena odorata plants or Gulma siam known in some areas with Kirinyu, Babanjaran, Darismin (Sunda), laruna, lahuna, kopasanda (Makassar) (Soerjani, et al., 1987 in Ward, 2006), spread almost all over the archipelago, both in the tropics and subtropics, reproduce very quickly. This plant is very rarely consumed by insects, including pets, tasted very bitter (Akoubundu 2010 in Riki, 2010).

Figure 2.1 Gulma siam Plant (*Chromolaena odorata*)

Classification of Gulma siam plants (Fox, 1990 in Ward, 2006):
Class : Magnoliopsida
Sub Class : Asteridae
Order : Asterales

Family : Asteraceae
Genus : Chromolaena
Species : *C. odorata* L.R.M. King and H. Robinson

This plant grows upright with a height of 2-6 m tall, trunk cylindrical shaped with color yellowish green with fine hairs, soft textured when young and the more mature the harder. Single flower colored mauve to whitish, in pile of bunches can reach 20-35 flowers (Abdullah, 2002 in nurhayati, 2006).

Gulma Siam plants (*C. odorata*) in some areas are used as a medicine of a new wound and as an ulcer medicine, becomes the first solution when getting wound to avoid infection. Several strands (to taste) *C. odorata* leaves are kneaded by hands until the extract is out then squeezed and applied or affixed the extract on the wound, it turned out to a very potent, and the wound dried. For patients with gastric inflammation (ulcer), simply by boiling the *C. odorata* plant, and drink its boiled water. Some people use it as a painkiller pain in the joints. Some countries in Asia, utilizing *C.odorata* as medicine, including Vietnam used in the treatment of wounds (new wounds, old wounds, burns) and other skin infections.

This plant contains variety of compounds that are antioxidants include tannins, flavonoids, alkaloids, steroids and triterpenoids (Yunilas, 2010), the possibility of the presence of those compounds that cause drug efficacy.

TOOLS, MATERIALS AND PROCEDURES

1. Tools

The tools used in the extraction step and identification include: evaporator, flash column, a regular funnel, Buchner funnel, Erlenmeyer flasks of various sizes, measuring cups, maceration vessel, capillary tube, spray bottles, tweezers,, UV light (wavelength 254 nm and 365 nm), hot plate, Pasteur pipette, scales, water bath, oven, chamber, vial bottle, stirring rod, FTIR spectrophotometer.

2. Materials

Gulma Siam leaves, methanol, n-hexan, acetone, ethyl acetate, acetone, kloroforom, stain apparition reagents Liebermann-Buchard, Dragendorff, and Wagner, 10% sulfuric acid, iron (III) chloride, Whatman filter paper, aluminum foil, tissue, silica gel 60 H Merck and silica gel G 60 (230-400 mesh) and aluminum KLT plate coated silica gel 60 G F254.

3. Procedure

a. Extraction

Fine powdered leaves of C. odorata macerated with methanol for 3 x 24 hours. The extract obtained was concentrated using a rotary evaporator to about a quarter of the initial volume (thick extract). The extract was then extracted with a non-polar solvent that is n-hexane to separate the non-polar compounds from the methanol extract, using separation funnel.

b. fractionation

n-hexane extract was then concentrated produce viscous extract with dark green color, then is thin layer Chromatography (TLC) with the mobile phase (eluent) that is varied they are; ethyl acetate: acetone; ethyl acetate: n-hexane; acetone: methanol to find the right mobile phase on vacuum liquid column chromatography (VLCC). On VLCC using stationary phase silica gel 60 H and the combined ethyl acetate: n-hexane, as the mobile phase in its polarity continuously improved, produced 20 fractions.

Fractionation results was TLC again with eluent ethyl acetate: n-hexane, and the fractions that have the same rf values combined, obtained with eluent ethyl acetate: n-hexane (4: 6) that were grouped into 3 combined fractions based on its chromotogram similarity that was A fraction (1-4 fraction) = 2.0 g, B fraction (5-9 fraction) = 1.4 g, and C fraction C (10-20 fraction) = 0.9 grams.

The C fraction is solid in a brownish green color weighing 0.9 g, tested with

Liebermann-Burchard reagent (terpenoids and steroids), FeCl3 (flavonoids), Dragendorff and Wagner (alkaloids). Then fractionated with flash column chromatography using silica gel G 60 H Merck as the stationary phase, and n-hexane 100%, ethyl acetate : n-hexane, and ethyl acetate 100% as the mobile phase.

Fractions obtained were analyzed with TLC with silica gel 60 GF254 as the stationary phase and eluent ethyl acetate : n-hexane as the mobile phase. The fractions that have the same rf values combined then evaporated to obtain a solid. Furthermore, the solubility test is done against some solvents such as n-hexane, chloroform, acetone, ethyl acetate and methanol.

c. Purification

The solid component obtained was washed with solvent n-hexane and then recrystallized using ethyl acetate solvent. The purity of the compounds obtained were determined by performing TLC systems three eluent they are chloroform : n-hexane (7: 3), ethyl acetate : n-hexane (9: 1), and acetone : n-hexane (9: 1) and then continued to the melting point test.

d. Identification

Identification of secondary metabolites compound from crystals obtained was done in two ways, they are:

1. Using reagent to determine the class of secondary metabolite compound that is Liebermann-Burchard for steroids and terpenoids, $FeCl_3$ for flavonoids, and Wagner and Dregendorf for alkaloids test.
2. Identification of functional groups with IR spectroscopy at the Laboratory of Chemistry FMIPA IPB.

RESULTS AND DISCUSSION

The extract obtained was fractionated by vacum liquid chromatography column (VLCC) using silica gel 60 H adsorbents as the stationary phase and 100 ml of n-hexane eluent that was enhanced its polarity with ethyl acetate as the mobile phase. The amount of eluent used in VLCC caused by on the first eluent its stain movement is still not good enough so the eluent used have to be increased its polarity. Fractionation with VLCC obtained 20 fractions as seen in Table 4.1, then chromatographed thin layer used eluent ethyl acetate : n-hexane (4: 6).

Based on the similarity stain on its chromatogram that is shown in Figure 4.1, The VLCC result fractions was combined till obtained 3 fractions combined as shown in Table 4.2.

Table 4.1 Observation Results of VLCC Fractionation

Fraction	eluent	Solution Color
	Ethyl acetate: n-hexane	
1	n-Heksan 100%	Yellow
2	1:19	Yellow
3	1:9	Yellow
4	1:9	Brown
5	2:8	Brown
6	2:8	Brown
7	2:8	Dark Green
8	3:7	Dark Green
9	3:7	Dark Green
10	4:6	Dark Green
11	4:6	Light Green
12	5:5	Light Green
13	5:5	Light Green
14	6:4	Greenish Yellow

15	6:4	Greenish Yellow
16	7:3	Greenish Yellow
17	7:3	Greenish Yellow
18	8:2	Greenish Yellow
19	9:1	Greenish Yellow
20	Ethyl acetate 100%	Greenish Yellow

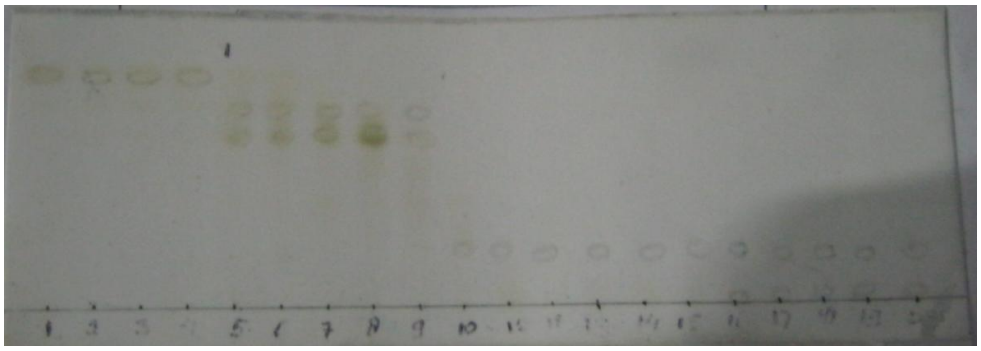

Figure 4.1 TLC chromatogram fractions VLCC eluent: ethylacetate: n-hexane (4:6), adsorbents: silica gel GF254, stain apparition: CeSO4 2%

Table 4.2 Results of Merger and Observation of VLCC fractions

Range of Fractions	Combined fractions	Mass (Gram)	Solids Color
1-4	A	2,0	Brownish Green
5-9	B	1,40	Brownish Yellow
10-20	C	0,90	Brownish Yellow

The C fraction shaped of solid brownish green color with weighing 0.9 g of was done a reagent test and than TLC.

FeCl3 reagent which showed negative results because no change of color.

Wagner and Dragendorf reagent gave a positive result against the alkaloid, is marked by a color change from brownish green to orange for Dragendrorf and the presence of a brown precipitate for Wagner. And positively to Liebermann Burchard that was marked by a change of color from brown to green.

The C fraction is not dissolved in the solvent n-hexane, but dissolved in polar solvents such as acetone, even dissolved completely in the solvent ethyl acetate, chloroform and methanol. This matter showed that the compound contained in fraction C is polar.

TLC test was then performed with eluent n-hexane: ethyl acetate and ethyl acetate: chloroform with a variety of comparisons, obtained eluent ethyl acetate: n-hexane 6: 4 by the apparition of stains CeSO4 2% after heating showed the appearance of stains with good separation pattern as in Figure 4.3. Based on that matter fraction C was fractionated further using eluent ethyl acetate: n-hexane 6: 4.

2. Results of Vacum Column Flash Chromatography Fractionation .

The combined of C fractions wighing 0.9 grams fractionated through flash column chromatography with silica gel G 60230-400 mesh as the stationary phase and eluent started from n-hexane 100%, ethyl acetate: n-hexane (6: 4), ethyl acetate 100% as the mobile phase resulted 33 fractions then continued with KLT analysis with developer eluent ethyl acetate: n-hexane (6: 4).

Table 4.3 Observation result of flash column chromatography.

Fractions	Eluent	Color
1	n-hexane 100%	Green
2 – 4	Ethyl acetate: n-hexane (6:4)	Blackish Green
5 – 9	6 : 4	Brownish Green
10 – 17	6 : 4	Yellowish Green
18 – 25	6 : 4	Yellowish Green
26 – 30	6 : 4	Clear Greenish
31 – 33	Ethyl acetate	Clear

Based on KLT analysis (Figure 4.4) from the 33 fractions, fractions that have the same chromatogram merged until it was obtained six fractions combined they are combined fractions Ca (fraction 1), Cb (fraction 2), Cc (fraction 3), Cd (fraction 4-7), Ce (fraction 8-19), Cf (fraction 20-33).

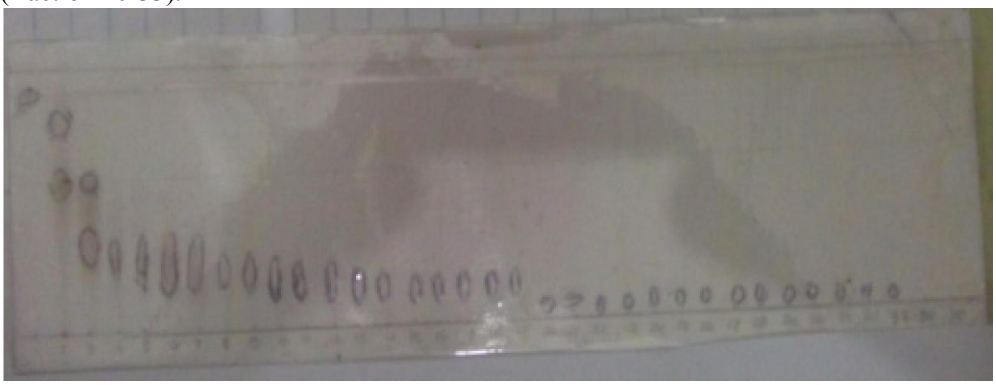

Figure 4.4 Chromatogram 33 fractions results from the chromatography flash column

Eluent : ethyl acetate: n-hexane (6: 4)
Adsorbents : silica gel GF234
Stains apparition : CeSO4

Fractions obtained were evaporated to one-fourth from its initial volume and then performed KLT analysis to combine fractions with the same Rf. From the result of Merging it was obtained 6 fractions they were Ca, Cb, Cc, Cd, Ce, Cf. After all of the fractions evaporated fraction that showed crystals was the fraction Cd.

3. Purification Result

The combined fraction 4-7 (Fraction Cd) showed the presence of crystals so potentially to be continued. After being evaporated the combined fraction Cd shaped crystalline amorphous with green color.

The amorphous-shaped crystals were then washed with n-hexane resulted white crystals with a weight of 30 mg then recrystallized with ethyl acetate resulted white crystals with a weight of 10 mg.

The C-d crystals were then purified with three eluents system test, that were chloroform: n-hexane (7: 3) with rf value of 0.325 (a), ethyl acetate: n-hexane (9: 1) with rf value 0.5 (b) and acetone: n-hexane (9: 1) with rf value of 0.825 (c), where the results showed a single kromotogram that can be seen in Figure 4.5.

4. Identification Results

The crystals obtained were identified including by:

a. Reagent test / color test with FeCl3 reagent, Liebarmann Burchard, Wagner, and Dragendroff. Cd crystals positive to Dragendorff reagent and Wagner. In the C-d crystal

there was a color changed to orange on Dragendroff reagent and brown (there are brown sediment) on Wagner reagent as seen in Figure 4.6.

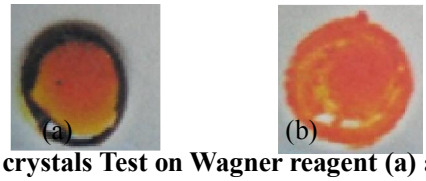

Figure 4.6 The result of C-d crystals Test on Wagner reagent (a) and Dragendorff reagent (b)

Untuk mengetahui kepolaran dari kristal C_d maka dilakukan uji kelarutan. Pada uji kelarutan kristal C_d tidak larut dalam n-heksan, larut dalam aseton, etil asetat, kloroform dan methanol. To know the polarity of th C-d crystal then solubility test was done. In the solubility test, C-d crystals were not soluble in n-hexane, soluble in acetone, ethyl acetate, chloroform and methanol.

b. On the melting point test it was obtained crystal Cd melting point at 144-145 oC. From this test, it can be seen the purity of a compound. If the compound has a melting point with a low trajectory then the compound can be categorized as a pure compound because if the trajectory is high then in that compound still contained impurities.

c. The results of IR spectroscopic measurements of C-d crystals.

Infrared spectroscopy (IR) can be used to identify a compound that has not been known because the spectrum produced is specific to the compound. Chromatograms IR spectroscopy results of C-d crystals can be seen in Figure 4.7.

Table.4.4 IR spectrum analysis results on the Gulma Siam leaves (*C. odorata*).

NO.	Wave number cm-1 on Gulma Siam leaves (*C. odorata*)	Cluster
1	3329,14	N-H
2	2916,37	C-H Aliphatic
3	1620,21	C=C Aromatic
4	1465,9	$–CH_2$
5	1357,89	C-N
6	1068,50	C-O

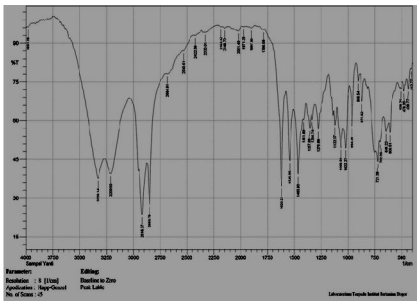

Figure. 4. 7. Infrared Spectrum of C-d Crystal

B. Discussion

1. Fractionation of flash column chromatography

The C Fraction (0.9 g) in the form of a brownish green solid of a VLCC result was a further analyzed fractions. Reagent FeCl3 test for C fraction showed negative results where there were no changes in color, which indicated that the fraction C contained no flavonoids.

For Wagner and Dragendroff reagent test gave a positive result on the alkaloid with color change from brown to orange at Dragendroff reagents and there was a brown precipitate on Wagner reagent. In Lieberman Burchard reagent showed positive results against steroid with

a color change from brown to green, this is due by the time of merger fractions, some fractions that contained in C fraction were intersecting with B fraction. In the solubility test the C fraction was not soluble in n-hexane, slightly soluble in acetone and completely dissolved in ethyl acetate, chloroform, and methanol (Table 4.3), this was indicated that the compounds contained in fraction C is polar. This fraction has a pretty good TLC separation result. The C fraction with eluent ethyl acetate: n-hexane (4:6) first analyzed with TLC before flash column chromatographed using several comparisons of different eluent. TLC itself aims to find out the number of components compounds still contained in C fraction that can be seen from the number of stains that appear on the chromatogram.

Moreover, TLC also aims to find out the best eluent for flash column chromatography. From the TLC test gave result that the most good eluent for use on flash column chromatography was ethyl acetate : n-hexane eluent (6: 4).

2. Purification

The C-d fraction (green color crystals and amorphous shaped) were washed with n-hexane then recrystallized using ethyl acetate to obtain more pure results (white color crystals and amorphous shaped) with a weight of 10 mg and showed single chromatogram of three eluent system with varied polarity level from relatively less polar eluent to relatively polar eluent.

The rf value is greater in relatively polar eluent that are chloroform: n-hexane (7: 3) with rf values 0.325, ethyl acetate: n-hexane (9: 1) with rf values of 0.5 and acetone: n-hexane (9: 1) with rf value of 0.825. This was indicated that the compounds obtained are relatively polar compounds, in accordance with the solubility properties of a compound based on polar solvent with dissolved compounds that is if polar solvent enhanced then the solute compound rise in accordance with the increase of its solvent means that the compound is a polar compound.

Table4.5 The Results Identification Test of C-d Fraction

Test										
Solubility					Reagents				IR	
C6H6	Aceton	CHCl3	CH3C2H5	CH3OH	LB	DG	Wagner	$FeCl_3$	Absorption area (cm^{-1})	Functional Groups
-	+	+	+	+	-	+	+	-	3329,14	N-H
									2916,37	C-H Aliphatic
									1620,21	C=C Aromatic
									1465,9	$–CH_2$
									1357,89	C-N Amin
									1068,50	C-O
Alkaloids										

3. Identification

Identification result of C-d Crystal was positive on Wagner reagent that indicated a brown precipitate and also positive on Dragendroff reagent (Table 4.5) that gave color changed from clear to orange.

This was indicated that the crystal was alkaloid positive. The melting point was 144-145^{O}C that showed a sharp trajectory. For the solubility test of the C-d crystals was not soluble in n-hexane, but dissolved in ethyl acetate, chloroform, acetone and methanol (Table 4.5)

Table 4.5 Identification Results (solubility test, reagents and IR) Cd crystal

IR absorption spectra that widened in the area 3329.14 cm^{-1} indicated the presence of N-H group, strengthened by the presence of CN absorption in the area 1357.89 cm-1. Sharp absorption in the area 2916.37 cm-1 indicated the presence of aliphatic C-H group, strengthened by the presence of absorption in the area 1465.9 cm^{-1} indicated the presence of-CH2- bending. Whereas medium absorption was at the area 1620.21 cm^{-1} was suspected absorption from aromatic C=C group. For absorption in the area 1068.50 cm-1 was suspected absorption from C-O group.

Based on the interpretation of data obtained from infrared spectrum (IR), positive on the color test using reagent Dragendorf and Wagner, not soluble in n-hexane, but soluble in polar solvents such as ethyl acetate, chloroform, acetone and methanol as well as the literature search then it can be concluded that crystal Cd that was obtained suspected to be alkaloid class compound.

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