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Research Article

ISOLATION AND IDENTIFICATION OF CHEMICAL COMPOUNDS IN BUTANOL FRACTION OF POHPOHAN (*PILEA TRINERVIA* ROXB.) LEAVES

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ABSTRACT

Pohpohan (*Pilea trinervia* Roxb.), Urticaceae, is a plant known as a raw vegetable salad (lalab) in West Java. This plant has many uses and benefits, but scientific research on the content of its chemical compounds is still limited. The purpose of this study was to isolate the chemical compounds contained in n-butanol fraction of pohpohan leaves. The leaf simplicia was macerated with 70% ethanol. Ethanol extract was fractionated using n-butanol and water solvents. n-butanol fraction was purified by preparative thin layer chromatography using the n-butanol: acetic acid: water (4: 1: 5) developer, producing N_{1-3} isolates (green at UV 366nm). The ultraviolet spectrum N_{1-3} showed the λ maximum wavelength at 208nm, and the infrared spectrum showed the presence of O-H groups, double bonds C = C, and C-O carbonyl. Analysis with gas chromatography-mass spectrometry (GC-MS) showed a fragmentation pattern of m / z: 180, 152, 151, 137, 95, 69, 57, 43. Based on fragmentation patterns, isolates were thought to have similar patterns with 10-methyl-9-oxabicyclo compounds [6.4] dodecane-1 (8) -en with 83% similarity index.

Keywords: Pohpohan, *Pilea trinervia*, n-butanol, 10-methyl-9-oxabicyclo[6.4]dodecan-1(8)-en, GC-MS

INTRODUCTION

Pohpohan leaves (*Pilea trinervia* Roxb.) are consumed by Indonesian especially Sundanese people as lalab (raw vegetable salad) [1]. Pohpohan is a plant that grows upright with a height reaching 1-2 m. In general, these plants grow in mountainous areas, namely in areas with an altitude of 600-2700 m above sea level. So far there has been no other use than as fresh vegetable [2-3]. Indonesia is an agrarian country that produces a lot of plant-based food, including vegetables that are consumed raw or made into vegetables by the people of Indonesia, especially in West Java. Traditionally brooders are known to have additional benefits for the body. Some negligence is believed to have certain properties, for example as a traditional medicine for diarrheal diseases, constipation, sweet blood, arteries and cancer [4].

Clinical testing of the efficacy of leaf pohpohan has been carried out and it is proven that the ethanol extract of the leaves of pohpohan and tespong (*Oenanthe javanica* DC.) has anti-cholesterol activity [5]. The results of pohpohan leaf simplicia phytochemical screening showed the presence of steroid/triterpenoid compounds, alkaloids, and flavonoids. From the ethyl acetate fraction, isolates are obtained namely alkaloid compounds containing groups> NH, -CH3, -CH2,> C = O,> C = C <and have conjugated double bonds [6]. In some species that have the same genus as pohpohan, their activity has also been tested. Three sesquiterpenes and one copaborneol derivative compound were isolated from the methanol extract of *Pilea cavaleriei* subsp. Crenata. The structure has been elucidated and cytotoxic has been evaluated [7]. Ethanol extract from *Pilea microphylla* (L.) fractionated with acetone, the fraction has been tested to have antioxidant activity and radioprotective effects [8].

Information about the content of chemical compounds in pohpohan leaves is still very little, so it is necessary to isolate and identify the chemical compounds contained in pohpohan leaves to further develop into nutritious medicinal plants.

MATERIALS AND METHODS

Plant material: Pohpohan leaves that had been dried and obtained from the Manoko Lembang plantation, and determined in the Taxonomy Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Chemicals: The chemicals used are amyl alcohol, ammonia, hydrochloric acid, aquadest, glacial acetic acid, 70% ethanol, ethyl acetate, chloroform (Merck), methanol (Merck), n-butanol (Merck), n-hexane, sodium hydroxide, TLC silica gel 60 F254 (Merck), magnesium powder, and silica gel 60 F254 plates for preparative TLC (Merck). Reagents used were: Mayer, Dragendorff, iron (III) chloride, 1% gelatin solution, vanillin-sulfuric acid, ether, and Liebermann-Buchard. All chemicals used were analytical grade

Instruments: Analytic balance (Mettler), macerator, rotary evaporator (Buchi), separating funnel, UV 254 and 366 nm lamps (Camag UV-Betrachter), vessels for developing thin layer chromatography (Camag), ultraviolet spectrophotometers (Specord 200-Analytic Jena), Infrared spectrophotometer (FT-IR 8400 Shimadzu), and Gas chromatography-mass spectrophotometry (Shimadzu QP-5050A).

Methods: The research method used was laboratory research methods including the collection preparing in the form collecting pohpohan leaves obtained from Manoko plantation, Lembang,

West Java and determined in toxicology laboratory, Biology Department, FMIPA, Universitas Padjadjaran. Pohpohan leaves were made simplicia referring to the method of making simplicia from the Indonesian Ministry of Health [9] and made a powder which was then macerated with 70% ethanol which refers to Materia Medika Indonesia [10]. The thick extract obtained after evaporation using a rotary evaporator and water bath, then fractionation referred to the Rahmawati and Mustarichie method [11] water, ethyl acetate, hexane, and n-butanol. The Farnsworth method [12]was applied for secondary metabolites of ethanol extract and n-butanol fraction. Detection of alkaloids by dissolving in 2N Hydrochloric acid and filtered. The filtrate was divided into four portions to achieve Dragendorff's test, Mayer's test, Wagner's test, and Hager's test; detection of flavonoids by treating with a few drops of sodium hydroxide solution which became colorless on addition of dilute acid, indicated the presence of flavonoids; detection of tannins by Gelatin test, Braymer's test, and Ferric chloride-potassium ferricyanide test; Froth test was used to detect saponins, crude dry powder of extract was vigorously shaken with 2 mL of distilled water and was allowed to stand for 10 min. If stable froth appeared, it indicated the presence of saponins; the present of quinones was detected by adding about 5 mL of 10 % ammonium hydroxide solution to the filtrate, shaken and allowed to stand till the two layers were separated. The development of pink, to violet color in the ammonical phase indicated the presence of free quinones; detection of triterpenoid by Libermann-Burchard test: The extract sample was dissolved in 2 mL of chloroform in a dry test tube. 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid were then added. If the solution became red, then blue and finally bluish-green in color, it indicated the presence of steroidal nucleus while the formation of purple or red color indicated the presence of a triterpenoidal nucleus. The ethanol extract of pohpohan leaves was analyzed by its components by thin layer chromatography, using the stationary phase of silica gel 60 F_{254}, n-hexane: ethyl acetate (7: 3) eluent, and spotting with UV light detection of 366 nm, 254 nm, and H2SO4 10% in ethanol. Then-butanol fraction of pohpohan leaves was analyzed by its components by thin layer chromatography, using the stationary phase of silica gel 60 F_{254}, developer of n-butanol: acetic acid: water (4: 1: 5), and detection of 366 nm UV light, UV light 254 nm, the appearance of vanillin-sulfuric acid spots. The n-butanol fraction of pohpohan leaves was analyzed by its components by thin layer chromatography, using the stationary phase of silica gel 60 F_{254}, developer of n-butanol: acetic acid: water (4: 1: 5), and detection of 366 nm UV light, UV light 254 nm, and the appearance of vanillin-sulfuric acid spots. To n-butanol fraction fraction was then eluted with a vacuum column chromatography (VCC) by eluent with a level polarity [13, 14] that would get 11 subfractions; Each subfraction was checked by two-dimensional thin layer chromatography to see subfraction purity characterized by the appearance of a spot on the chromatogram plate. This subtraction was a selected isolate. The isolate was then identified using ultraviolet-visible spectrophotometry, IR-spectrophotometry, and GC MS.

RESULTS

Table 1: Phytochemical Screening results of ethanol extract and n-butanol fraction

Secondary metabolites	Ethanol extract	n-butanol fraction
Alkaloids	+	-
Flavonoids	+	+
Tannins	-	-
Polyphenols	-	-
Monoterpenoid and sesquiterpenoid	+	+
Steroids	+	-
Triterpenoids	-	-
Quinone	-	-
Saponins	-	-

Notes: (+) detected; (-) not detected

Table 2: TLC Ethanol Extract of pohpohan leaves with n-hexane: ethyl acetate (7: 3) developer

Spots	R_F	Visible light	UV		H_2SO_4 10% in Ethanol
			254 nm	366 nm	
1.	0.83	Green	-	Bright pink	-
2.	0.78	-	-	Orange	-
3.	0.70	Green	-	Pink	Pale pink
4.	0.64	Yellowish green	-	-	Pale pink
5.	0.55	Yellow	-	Pink	-
6.	0.50	-	-	-	Purple
7.	0.45	Dark green	-	Bright pink	Purple
8.	0.36	Yellow	Purple	-	Hijau
9.	0.33	-	-	Pink	-
10.	0.25	Yellow	Yellow	Pink	Green
11.	0.20	-	-	-	Yellowish green
12.	0.15	-	Purple	-	-
13.	0.11	-	Yellow	-	-
14.	0.09	Green	-	Pale pink	-

Table 3: TLC of n-butanol fraction

Spot	Rf	UV 366 nm
1	0.96	Red
2	0.64	Light blue
3	0.53	Green

Table 4: Preparative TLC of n-butanol fraction

Pita ke-	Rf	UV 366 nm
N_{1-1}	0.94	Red
N_{1-2}	0.72	Light blue
N_{1-3}	0.48	Green

Table 5: Two-way TLC of n-butanol fraction

Direction of development	Rf	UV 366 nm
1	0.75	Green
2	0.52	Green

Table 6: IR Results of N_{1-3} isolate

Wave numbers (cm^{-1})	Shape	Intensity	Estimation
3328.50	width	strong	O-H bending
1571.00	sharp	strong	C-H bending
1417.87	sharp	strong	C=C stretch
1025.69	width	weak	C-O bending
675.57	width	weak	C-H bending

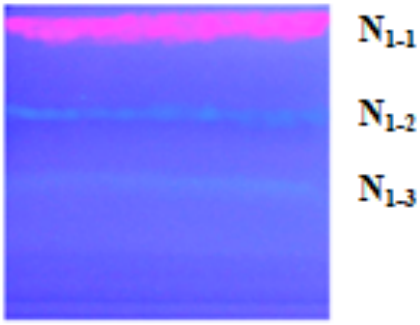

Fig 1: Results of preparative TLC

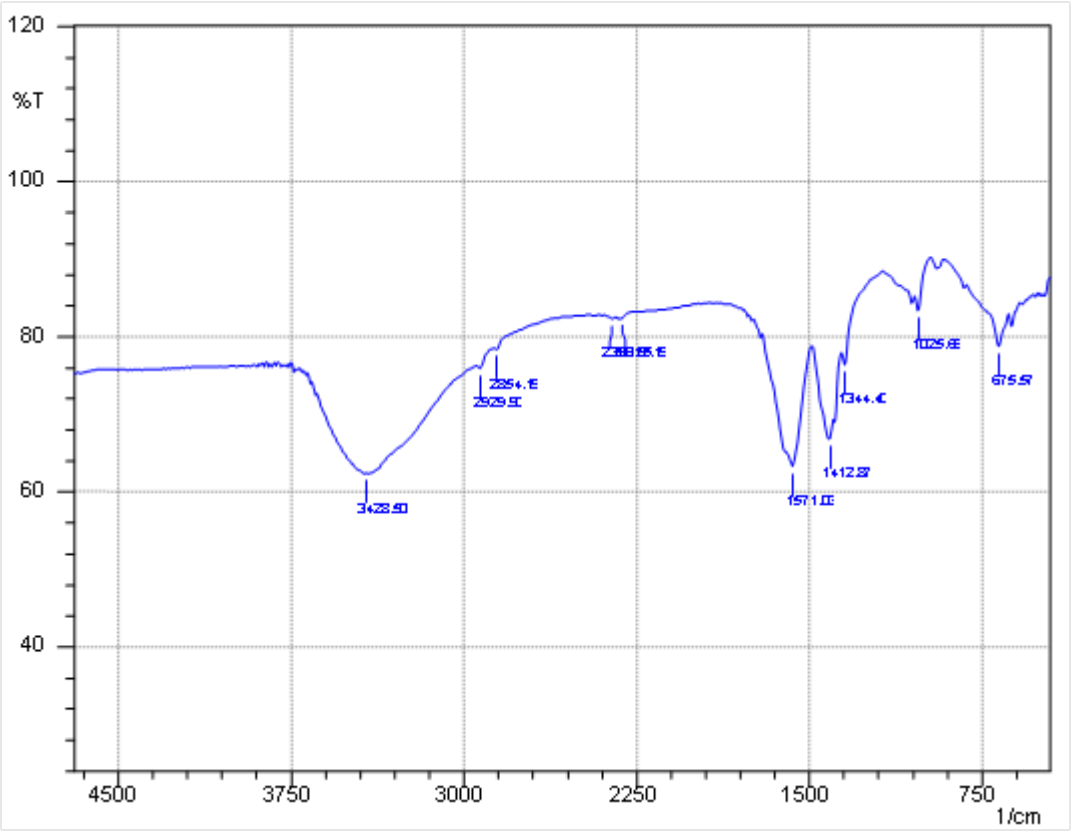

Fig. 2: IR spectra of N_{1-3} isolates

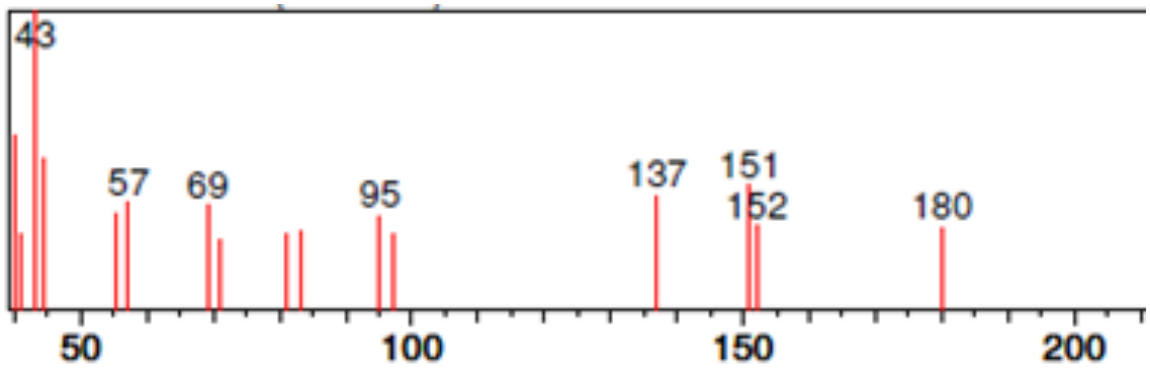

Fig 3: The mass spectrum of N_{1-3} isolate

DISCUSSION

Plant Determination: The results of plant determination at the Laboratory of Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung showed that the plants used in the study were pohpohan (*Pilea trinervia* Roxb.).

Extraction: A total of 1170g of dried pohpohan leaves were extracted by maceration using 70% ethanol. The ethanol extract produced was dark green. The ethanol extract was then rotary evaporated and obtained 147.45g of thick ethanol extract with a percent yield of 12.60%. The extraction method chosen was maceration. This cold extraction was used to prevent changes in unwanted chemical compounds. The maceration process was carried out by soaking the simplicia with the fluid while stirring occasionally to evenly concentrate the solution. Stirring allowed fresh solvents to flow repeatedly into the entire simplicia surface so that there was an equilibrium concentration between the solution in the cell and outside the cell [15]. The liquid used in the maceration process was 70% ethanol because, in addition to fulfilling the general criteria of the liquid sealing, 70% ethanol could also attract almost all compounds from pohpohan leaves. This extraction process was repeated three times to maximize the withdrawal of compounds. The effectiveness of the extraction process would be determined by the number of repetitions of the extraction process. Repeated extraction would be more effective than one-time extraction [16]. Ethanol extract was then concentrated carried out by a rotary evaporator at 40°C to avoid damage to the components of chemical compounds contained in the extract, especially the thermolabile components. The concentrated extract was then collected into separate vaporizer plates and heated in a water bath to evaporate the remaining solvents in the extract. The ethanol extract produced was dark green. It should be underlined that the advantage of the method of using the maceration method was how to do it and the equipment used was simple and easy to do. While the disadvantages of maceration were the long processors and their lack of perfection [17].

Fractionation: At this stage the principle of like dissolves like applies, meaning that the compounds contained in the extract would be attracted by solvents with relatively similar polarity. This process was carried out to separate the compounds contained in the extract according to their polarity so as to facilitate the analysis process. Extraction was done repeatedly to optimize the separation, then the n-butanol fraction was concentrated with a rotary evaporator. Fractionation was carried out on ethanol extract of pohpohan leaves using liquid-liquid extraction method using n-hexane, ethyl acetate, n-butanol, and water. The water fraction was brown and the n-butanol fraction was brownish yellow in color. From 1170 g of dried pohpohan leaves, an n-hexane fraction of 20.04 g was produced with percent yield of 1.713%, ethyl acetate fraction of 12.19 g with percent yield of 1.042%, and the n-butanol fraction of 7.68 g with percent yield by 0.66%.

Phytochemical Screening: Phytochemical screening of extracts was carried out to determine the secondary metabolites contained in ethanol extract and n-butanol fraction of pohpohan leaves. This phytochemical screening was carried out on alkaloid compounds, flavonoids, tannins and polyphenols, saponins, monoterpenoids and sesquiterpenoids, steroids and triterpenoids and quinone compounds. Table 1 showed a phytochemical screening of ethanol extract and n-butanol fraction. Up to now, there had been no publication regarding the results of phytochemical screening of the n-butanol fraction of *P.trinervia*. Iskandar and Mustarichie [18] stated that ethyl acetate fraction from *P.trinervia* contained alkaloids, mono and, and steroids, but these secondary metabolites were not found in the n-butanol fraction.

TLC of ethanol extract of *P.trinervia*: Thin layer chromatography (TLC) analysis of pohpohan leaf ethanol extract was carried out using the stationary phase of silica gel 60 F254 and n-hexane: ethyl acetate (7: 3) developer (see Table 2).

TLC of the n-butanol fraction of *P.trinervia:* Thin layer chromatography (TLC) analysis of the n-butanol fraction of pohpohan leaves was carried out using the stationary phase of silica gel 60 F254 and developer of n-butanol: glacial acetic acid: water (4: 1: 5) (see Table 3).

Preparative Thin Layer Chromatography: Preparative thin layer chromatography was a separation technique in which a rather thick absorbent layer (0.5-2.0 mm) was used. The band was proposed as a narrow line with the appropriate tool. Trailer bands ought to be as narrow as possible because bandwidth would affect separation. Spotting could be done manually using a pipette or automatic spotter. The maximum size of the trailer depended on the relative number of constituent compounds [6]. One of the advantages of preparative TLC was that it was inexpensive and could be done with a simple tool [19].

Preparative thin layer chromatography analysis of n-butanol fraction was carried out using n-butanol developer: glacial acetic acid: water (4: 1: 5) (Table 4). From the results of preparative TLC, three bands could be detected under UV light 366 nm (N_{1-1}, N_{1-2}, and N_{1-3}). This band was scraped and then placed into a vial bottle and dissolved with ethanol solvent. The N_{1-3} tape was analyzed using thin layer chromatography with developer n-butanol: acetic acid: water (4: 1: 5). This chromatographic result showed one green spot that could be seen under 366 nm UV light. To ensure the purity of these isolates, the analysis was carried out

using two-dimensional thin layer chromatography. The developer used was n-butanol: acetic acid: water (4: 1: 5) while the second developer was ethyl acetate: methanol (7: 3). Based on the analysis with two-way chromatography, one green spot was observed under UV 366nm. This indicated that pure N_{1-3} isolates (Fig. 1).

Isolate Purity Test: The isolate purity test was carried out by two-way thin layer chromatography (TLC) method. In this test two different developers were used, the first developer was n-butanol: acetic acid: water (4: 1: 5) while the second developer was ethyl acetate: methanol (7: 3). The two-way TLC performed on isolates N_{1-3} showed one green spot after 366 nm of UV light. In the first direction of development, a single green spot appeared under UV 366nm with Rf 0.75, and in the second development, a green single spot appeared under UV 366nm with Rf 0.52. (Table 5).

Isolate Identification: Identification of N1-3 isolates was carried out using ultraviolet spectrophotometry and infrared spectrophotometry. The ultraviolet spectrum of N_{1-3} isolates showed a maximum wavelength of 208 nm. This showed that the structure of isolate N_{1-3} contained a double bond.

The results of infrared spectrophotometry showed absorption in several wavenumbers. Infrared spectrophotometry results can be seen in Fig. 2 and Table 6. Infrared spectrophotometry could be used to identify functional groups found in compounds. Specific functional groups provided specific bands between 4000 to 1400 cm^{-1} wave numbers in the infrared spectrum. Wave numbers below 1400 cm^{-1} or fingerprint areas could also be used but were less specific to characterize compounds. The infrared spectrum of isolate N_{1-3} showed a band at wave number 3328.50 cm^{-1} with a strong intensity and a widening band which showed a flexible O-H, and at 1571.00 cm^{-1} with strong intensity showed a C = C strain in the isolate. The absorption band at 1417.87 cm^{-1} with strong intensity was thought to originate from bending C = C aromatic. The absorption band with a weak intensity at 1025.69 cm^{-1} was thought to originate from bending C-O. Based on these results it was suspected that the N_{1-3} isolate was a compound that had a double bond C = C and contained a carbonyl group.

Isolates were analyzed using the Shimadzu QP-5000 series KG-SM. Before being analyzed, samples were dissolved in ethyl acetate. The samples were then analyzed using the following conditions:

1. Gas Chromatography Conditions:
Carrier gas: Helium
Column: DB-Wax capillary column 30 m long, diameter 0.32 mm
Interface temperature: 300ºC
Detector temperature: 290ºC
Column pressure: 75 kPa
Flow rate: 1.6 mL per minute
Flow mode: Linear velocity 46.4 - splitless
Injection volume: 1 µL
The initial temperature: 60ºC was increased by 10ºC per minute
Temperature analysis: 300ºC was held for 2 minutes, increased by 10ºC per minute
Final temperature: 290 ° C held for 2 minutes

2. Mass Spectrometry Conditions:
initial m / z: 33
final m / z: 600
Scan interval: 0.5 seconds
Scan speed: 2000 amu / second

The reasons for using GC-MS in determining isolate compounds were as follows. Gas chromatography - mass spectrometry was a combination of two analytic techniques where gas chromatography was used as a method of separation while mass spectrometry was used as an identification method. This combination of analytical techniques produces several advantages, which could be used for analysis qualitatively and quantitatively, and the use of these two methods could overcome weaknesses that arise when used separately. The use of mass spectrometry without gas chromatography was difficult because the compounds used for analysis with this tool must be completely pure. If impure compounds were used the mass spectrum would be obtained which was complicated and difficult to interpret [20].

Analysis using mass spectrometry showed that isolate N1-3 had a fragmentation pattern m / z: 180, 152, 151, 137, 95, 69, 57, 43 (see Fig. 3). The isolate was thought to have a molecular weight of 180 with the molecular formula C12H20O and was thought to have a double C bond = C on the molecular structure. The double bond in the isolate was strengthened by the appearance of absorption bands at wave number 1417.87 cm-1 in the infrared spectrum. Based on fragmentation patterns, isolates have similar patterns with 10-methyl-9-oxabicyclo compounds [6.4] dodecane-1 (8) -en with a similarity index of 83%. Hardinsyah and Briawan [21] mentioned that the chemical content in pohpohan leaves (per 100 grams of ingredients) was energy 37 Kal, 2.5 gr protein, 0.8 gr fat, 6.9 gr carbohydrates, Calcium (Ca) 744.0 mg, 80.0 mg phosphorus, iron 5.9 mg, vitamin A 900 RE, vitamin C 5 mg, vitamin B1 0.03 mg, water 87.4 gr and BOD 69%. A total of 34 compounds were identified from *Pilea trinervia essential* oil which was reported by Wibowo and Mariani [22]. Their essential oil was obtained through water and steam destillation. So far there is no literature that states the chemical content of the n-butanol fraction of pohpohan leaves.

CONCLUSION

Based on phytochemical screening results, it was found that n-butanol fraction of pohpohan leaves (*Pilea trinervia* Roxb.) contained flavonoid and monoterpenoid/sesquiterpenoid compounds. From n-butanol fraction, N_{1-3} isolates were found which had a maximum wavelength at 208nm, the infrared spectrum showed that isolates had an O-H group, a double bond C = C, and a C-O carbonyl group. Analysis with mass spectrometry showed a fragmentation pattern of m / z: 180, 152, 151, 137, 95, 69, 57, 43. Based on fragmentation patterns, the isolates were thought to have a molecular weight of 180, C12H20O molecular formula, and similar pattern with 10-methyl compounds -9-oxabicyclo [6.4] dodecane-1 (8) -en with 83% similarity index.

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