International Journal of Applied Chemistry.
ISSN 0973-1792 Volume 12, Number 3 (2016) pp. 347-357
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Secondary Metabolites Identification From Lichen *Usnea longissima* Ach. : Bioactivity Test of Antibacterial

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Abstract

Isolation and identification of secondary metabolite compound from chloroform fraction of *Usnea longissima* and its bioactivity test as an antibacterial had been conducted. The isolation of the chemical compound was performed by using Gravity Column Chromatography (GCC) and Thin-Layered Chromatography (TLC). The result showed the needle crystal shaped isolated compound with translucent white color. The 1D-NMR (^{1}H and ^{13}C-NMR) data analysis and comparation of the similar data from the isolated compound literature was (5E, 6E) 5-ethylidene-7-formil-6,7-dihydroxy methyl hept-6-enoate. The antibacterial bioactivity test using the method of paper disc diffusion showed that the chloroform extract inhibited the bacterial growth at the concentration of 100 ppm, 250 ppm, 500 ppm and 1000 ppm for *E.coli* ATCC 35218, *S.auerus* ATCC 25923 and *S. typhi* YCTC. The isolated compound obstructed the bacterial growth at the concentration of 100, 250, 500, and 1000 ppm for *E. coli*, *S. Typhy* and it was inactive for *S.auerus*.

Keywords: Isolation, identification, *Usnea longissima*, antibacterial.

INTRODUCTION

Indonesia is a tropical country that is rich of various kinds of plants [1]. Approximately 30,000 species of plants are very helpful and some of them had been used by the people as the medicine ingredients and had been consumed sustainably and hereditary for a long time based on the traditional experiences [2]. The potention of biological diversity in Indonesia had been explored maximally so that they can be used as a natural medicinal compounds [3,4]. The natural compounds are usually

secondary metabolite produced by the metabolism process of plants [5,6]. The advantage of natural ingredients from the plants based on their structures are easily accepted by the body, it is because of the comformity among the reactions that occur in a series of metabolic cycle of human and plants [7]. One of the plants contained of natural medicinal compound is lichen [8]. Lichen is one of the pioneer plants that have approximately 100,000 species in the world. In Indonesia, lichen is used as a mixture of herbs, as natural herbs because it can cure with various diseases, such as anti-cancer, anti-fungi, antioksida, and anti-malaria [8,9].

There are approximately 350 chemical compounds having biological activity isolated from lichen and more than 200 compounds have been characterized [10]. The study of chemo-taxonomy shows that the secondary metabolit of lichen plants are from the group of depsida, depsidon, dibenzofuran, and xanthone [11]. Some of xanthone compounds have toxic activities against some types of cancer cells and anti-malaria activity against *P.falciparum* [12].

Lichen is used as the medicine ingredient related to its substances. Its substances are used for antibiotics, anti-fungi, anti-virus, anti-inflamation, analgesic, antipyretics, antiproliferative, and cytotoxic effects [13]. Other, lichen is an indicator species for air pollution, it is sensitive to toxic so that it is useful as an early warning indicator to monitor the environmental pollution [14,15].

Generally, most of the secondary metabolit of lichen has ability of inhibiting the activities of bacteria and fungi [16,17]. Atranorin isolated from *Cladonia foliacea* and *Usnea sp*. shows the activity of antibacterial either the type of gram-positive or gram-negative bacteria [4,18]. Chloroatranorin from *Pseudevernia furfuracea* can inhibit the activities of bacteria and yeast [19]. Lecanoric acid shows the activity of inhibiting bacteria and fungi [20]. Protolichesterinic acid from *Cetraria aculeate* shows the activity of antibacterial [21].

According to the previous reports, the biological bioactivity study of one of lichen, *Usnea longissima* from Indonesia has not been known yet. Therefore, it is important to isolate and identify the secondary metabolic content structure of lichen from chloroform fraction *U. longissima* and its bioactivity test as antibacterial against *Escherichiacoli* ATCC35218, *Staphylocuccusaureus* ATCC25923 and *Salmonellatyphi* YCTC.

REASEARCH METHODS

1. Extraction and Partition

The lichen plant *U. longissima* were collected, washed, and dried by aerating them at a room temperature. Then, the plant was pulverized to powder. 710 grams sample powder of *U. longissima* and macerated with 5.5 L methanol for 3 x 48 hours. Each macerate from the sample was combined and concentrated by using rotary vacume evaporator.

It was continued to the process of partition by using three fractions, which are non-polar fraction, semi-polar fraction, and polar fraction. Beginning from the n-hexane solvent (non polar fractions), continuing to the methanol extract with the chloroform solvent (semi-polar fraction) by shaking and letting it until it was separated (the layer

was formed). The filtrate on the bottom layer (chloroform fraction) was turned out and the chloroform fraction was concentrated and weighed.

2. Separation and Purification

a. Thin-Layered Chromatography (TLC)

Concentrated chloroform fraction (1 mL) was dissolved into 2 mL of chloroform and dropped on the TLC plate by using capillary pipe. The eluent used for eluting the fractions was n-hexane (non-polar solvent) for the beginning, continued by the mixture of n-hexane and ethyl acetate (semi-polar solvent).

The eluent with the best ratio was used as a reference in the process of column chromatography. The analysis of the separation itself was done by using UV lamp and the stain performer reagent (Cerium Sulfate ($CeSO_4$)). The R_f of stain that appeared on the TLC plate was counted.

b. Gravity Column Chromatography (GCC)

Silica Gel G60 (p.a.) was weighed 20 times of the sample weight and was eluted by using n-hexane to condense the contents in the chromatography column. Then, the sample of chloroform fraction was weighed to be 2 grams and mixed with Silica Gel G. 60 (twice of the sample weight) and dissolved by using chloroform, stirred evenly until it was dry (impregnation).

The sample of impregnation result had been eluted continually by increasing the eluent polarity gradiently to be the most polar eluent. The result of all fractions was then tested on the TLC plate and the R_f (Retardation factor) values were counted for each fractions. The TLC result of each fraction was analyzed by using UV lamp and the stain performer reagent. The fractions with the same R_f value were joined together and concentrated.

3. Compound Identification

Compound structures of the obtained pure isolated compound were determined by spectrophotometry method using 1D-NMR (^{1}H-NMR and ^{13}C-NMR). The obtained data from the 1D-NMR instrument measurement were then interpreted by comparing them with the literature so that the structure of isolated compound could be determined.

4. Preparation of *Nutrient Agar* (NA) Media and Culture

The glass equipments were sterilized in an autoclave during 15 minutes on the temperature of 121°C at the pressure of 1 atm. Then, the Nutrient Agar (NA) media was made by dissolving 20 grams of NA in 1000 mL of distilled water and was heated and it was dissolved perfectly. Natrium Klorida (NaCl) 0.9% was prepared by weighing 0.9 grams of solid NaCl dissolved in 100 mL. Then, it was sterilized in autoclave at the pressure of 1 atm on the temperature of 121°C during 15 minutes. All the processes were sterilized in the autoclave so that they were not contaminated by another bacterial and remained steril. The test microorganisms used in the test of antibacterial activity consist of *E.coli*, *S.aureus* and *S.typhi*. Each bacterium was

rejuvenated by replacing 1 or 2 ose of the bacterial culture in the media, so that it could be oblique, to the light bottle containing NB liquid media and it was incubated for 24 hours at the temperature of 37 ± 2°C.

5. Test of Antibacterial Activities

The method of antibacterial test used agar diffusion with disc paper, which inoculated 1 mL of each bacterial suspension into 15 mL agar media that had been melted in a sterile petri dish and then was let to be solid. The disc paper that was dropped by each of the test material was placed on the surface of the media and left to stand for 30 minutes at the room temperature before being put into an incubator at the temperature of 37°C. The result of antibacterial test was based on the measurement of the inhibitory area diameter of bacterial growth around the disc paper.

RESULT AND DISCUSSION

A. Identification of Isolated Compound Structure

The isolated compound obtained was in the form of crystal translucent needle (Figure 1). This purified compound was then identified with some spectroscopy instruments to determine the structure of the compound obtained from the isolation process.

Figure 1. The isolated crystal of chloroform fraction of lichen *U. Longissima*

The compound structure was isolated from the lichen *U.longissima* and determined by using spectrophotometry ^{1}H-NMR and ^{13}C-NMR.

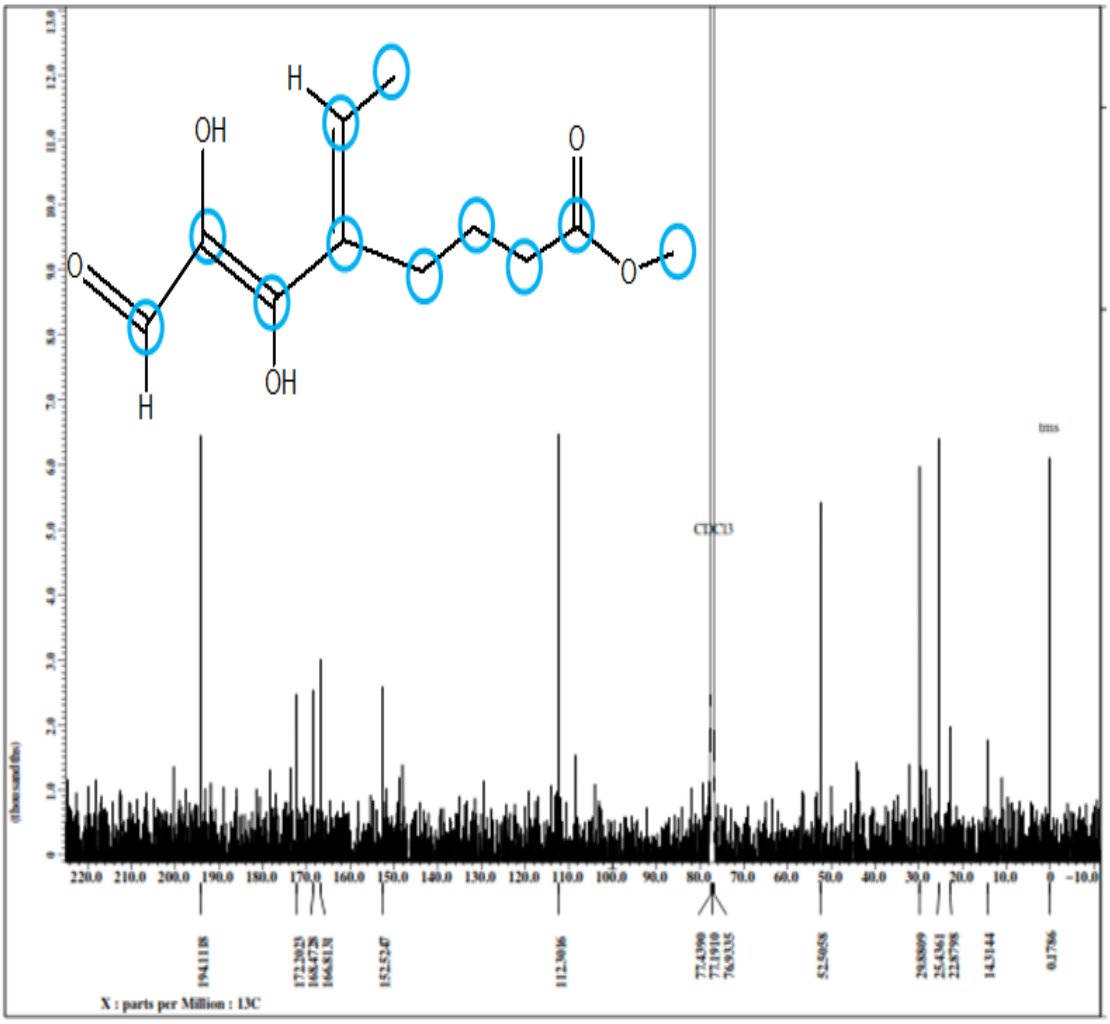

Figure 2. The spectrum of ^{13}C-NMR isolate compound

Note ◯ = karbon (C)

Spectrum ^{13}C-NMR showed the structure of 11 carbon constituent signals indicated that the isolated compound. Those 11 carbons included 1 carbon that had a relatively high chemical shift caused by the presence of carbonyl group (C=O), that were aldehyde carbonyl (δc 194.1118 ppm), 1 carbon atom sp^2 methine (C-H) (δc 112.3016 ppm), 1 quaternary carbon sp^2 (C=C=) (δc 152.5247 ppm), 1 carboxyl carbon atom (COO) (δc 172.2023 ppm), 3 secondary carbon atoms sp^3 (CH_2) (δc 29.8809 ; 25.431 ; 22.8798 ppm), 1 methoxy carbon (O-CH_3) (δc 52.5058 ppm), 2 carbons binding hydroxy group (C-OH) (δc 168.4728 ; 166.8131 ppm) and 1 carbon sp^3 methyl (CH_3) (δ_C 14.3144 ppm).

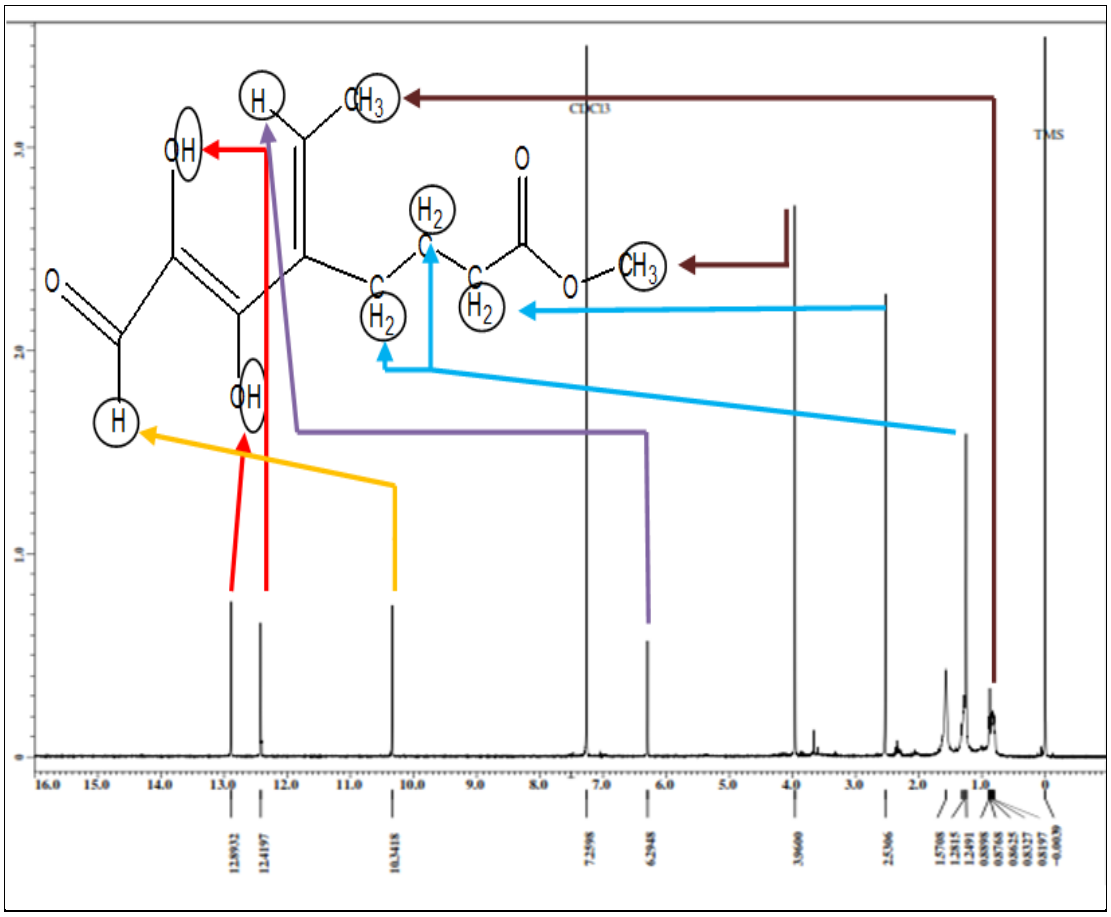

Figure 3. The spectrum of ^{1}H-NMR isolated compound

Notes:

= Aldehyde group protons
= Hydroxyl group protons
= Ethylene group protons
= Methylene group protons
= Methoxy and methyl group protons

The data ^{1}H-NMR showed that the isolated compound had 16 protons. The presence of 2 protons bonded to hydroxyl group that were bonded to carbon sp^2 was indicated on the shift (δ_H 12.8932, and 12.4197 ppm) appropriate with the signal ^{13}C-NMR, the shift (δ_H 10.34118 ppm) showed the protons bonded on the aldehyde groups. The presence of ethylene proton was showed on (δ_H 6.2948 ppm) indicating that the proton had low electron density or it was bonded to electron withdrawing groups, the shift (δ_H 3.9600 ppm) showed the presence of 3 protons from methyl bonded to carbonyl group, the shift (δ_H 0.8898 ppm) showed the presence of proton from methyl, and the presence of proton bonded to methylene group was showed on the shift (δ_H 2.5306, 1.2815 and 1.2491 ppm).

Table 2. The data of chemical shift of ^{1}H-NMR and ^{13}C-NMR isolated compound.

C Position	^{1}H-NMR of Isolate (ppm)	^{13}C-NMR of Isolate (ppm)	^{1}H-NMR References [22] (ppm)	^{13}C-NMR References [22] (ppm)
1		172.2023		175-185
2	2.5306 (2H, *t*,H-2)	29.8809	2.2-2.5	20-60
3	1.2491 (2H,*m,*H-3)	25.431	1.1-1.5	20-60
4	1.2815 (2H, *t*,H-4)	22.8798	1.1-1.5	20-60
5		152.5247		100-150
6	12.8932 (1H,*s,*OH)	168.4728	12	165-175
7	12.4197 (1H,*s,*OH)	166.8131	12	165-175
7'	10.34118 (1H*,s,*CHO)	194.1118	9.4-10.4	190-200
5'	6.2948 (1H,*m,H-5')*	112.3016	6.0-8.0	100-150
5''	0.8898 (3H, *d,*H-5'')	14.3144	0.8-0.2	8-35
1'	3.9600 (3H, *s*, Me-1')	52.5058	3.2-4.3	50-80

The data above showed that the isolate compound had the molecule pattern $C_{11}H_{16}O_5$ with DBE (*Double Bond Equivalence*) 4. The value of DBE was determined with the pattern F=X–½Y+½Z+1,F=11–½(16)+½(0)+1=4.

Notes:

F= The number of ring or double bond
X=The number of tetravalent atom
Y= The number of monovalent atom (H, F, B, Cl)
Z= The number of trivalent atom (N, P)

Four (4) as the value of DBE came from 1 carbonyl group, 1 carboxyl group and 2 double bond from the compound structure design (Figure 4).

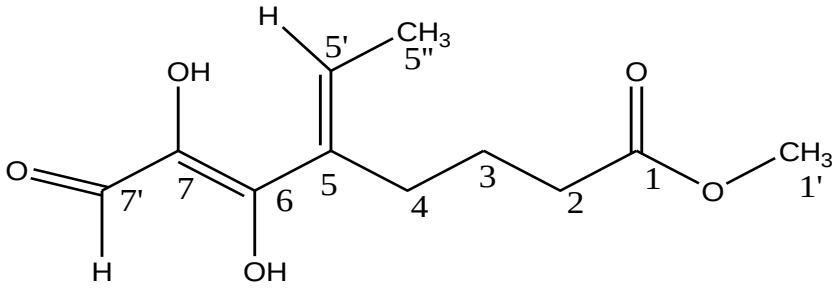

Figure 4. The structure of isolated compound (5E, 6E) 5-ethylidene-7-formil-6,7-dihydroxy methyl hept-6-enoate

According to the data of ^{13}C-NMR and ^{1}H-NMR, the compound (5E, 6E) 5-ethylidene-7-formil-6,7-dihydroxy methyl hept-6-enoate was proposed to be the compound isolated from lichen *U. longissima*.

C. Test of Antibacteria Activity of Chloroform Fraction and Isolated Compound

1. The Antibacterial Activity of Chloroform Fraction

The test of chloroform fraction activity to the bacteria was done triplo by the agar diffusion method using a disc paper with the diameter of 6 mm. The activity test was done to 3 kinds of bacterial, that are gram-positive bacterial *S.aureus* and the gram-negative bacteria *E.coli,* and *S.typhi.* The result of the test could be seen on the Table 3.

Table 3. The antibacterial test result of chloroform fraction

Bacterial Species	Diameter of inhibition zone (mm)					
	Chloroform fractions (mg/mL)				$CHCl_3$	Chloramphenicol (1000 mg/mL)
	100	250	500	1000		
E.coli ATCC 35218	1.3	2.1	2.7	3.4	0	20
S.auerus ATCC 25923	2.3	2.5	2.7	3.7	0	20
S. typhi YCTC	2.1	3.4	2.6	3.4	0	18

The diameter of translucent zone was not included in the diameter of the disc paper (6mm)

The result of antibacterial test of chloroform fraction showed that the chloroform fractions was active as an antibacterial showed by the forming of translucent zone on the media of *E.coli*, *S.auerus*, *S.typhi*. According to the result of antibacterial test by comparing the data of resistance response classification of the bacterial growth, the chloroform fraction had the weak response of the resistance growth to *E.coli*, *S.auerus*, *S.typhi*. In the chloroform fraction, there was not any kind of compounds that had the activity of antibacterial isolated from *U.longissima* lichen, the chloroform fraction produced the different compound so that the chloroform fraction had the antibacterial activity with the low resistance power.

2. Antibacterial Activity of Isolated Compound

The activity test of isolated compound to the bacterial was done triplo by the agar diffusion method using a disc paper with the diameter of 6 mm. The activity test was done to 3 kinds of bacterial, that are gram-positive *S.aureus* and the gram-negative *E.coli,* and *S.typhi.* The result of the test could be seen on the Table 4.

Table 4. The antibacterial test result of isolated compound

Bacteria Species	Diameter of inhibition zone (mm)					
	Isolate compound (mg/mL)				$CHCl_3$	Chloramphenicol (1000 mg/mL)
	100	250	500	1000		
E.coli ATCC 35218	3	4	5	5	0	20
S.auerus ATCC 25923	0	0	0	0	0	20
S. typhi YCTC	2	3	3	4	0	18

The diameter of translucent zone was not included in the diameter of the disc paper (6mm)

The test result of antibacterial showed that the isolated compound with the concentration of 100, 250, 500, and 1000 ppm could inhibit the growth of the bacteria with the resistance diameter of 3, 4, 5, and 5 mm for the *E.coli* after the reduction by the solvent control was used, and 2, 3, 3, and 4 mm for the *S.Typhy*. Compared to the chloramphenicol that could inhibit the *E.coli* with the resistance diameter of 20 mm and for the *S.Typhy* with resistence diameter of 18 mm, it could be concluded that the isolated compound from the chloroform extract of the lichen *usnea longissima* had a weak antibacterial activity. The result of antibacterial test also showed that the isolated compound was not active on *S.aureus*. The isolated compound could inhibit the growth of bacterial because it contained of alcohol groups that are bactericidal by damaging the tertiary structure of the bacterial protein or protein denaturation.

CONCLUSION

According to the previous result and discussion, it can be concluded that the plant isolation result of *U.longissima* lichen from the chloroform fraction and the identification using 1D-NMR (^{1}H-NMR and ^{13}C-NMR) spectrophotometer and by comparing the data from literature showed that the secondary metabolit compound was successfully isolated of (5E, 6E) 5-ethylidene-7-formil-6,7-dihydroxy methyl hept-6-enoate. The bioactivity test of the lichen *U.longissima* plant antibacterial by the diffusion method using a disc paper showed that the chloroform extract inhibit the growth of bacteria at the concentration of 100 ppm, 250 ppm, 500 ppm and 1000 ppm with the weak resistance power for the *E.coli* ATCC3521, *S.auerus* and *S.typhi* YCTC. The isolated compound inhibited the growth of bacteria at the concentration of 100, 250, 500, and1000 ppm with the weak resistance power for the *E.coli* ATCC35218, *S.Typhy* YTCC and was not active on *S.auerus* ATCC25923.

ACKNOWLEDGEMENT

We acknowledge for financial support of the DRPM-Ministry of Research, Technology and Higher Education, the Republic of Indonesia.

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