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Antibacterial activity of usnic acid from *Usnea longissima* Ach.

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Abstract: Isolation and identification of secondary metabolite from *n*-hexane fraction of lichen *Usnea longissima* Ach. and its bioactivity as antibacterial have been carried out. The isolation of chemical compounds was performed by using Gravity Column Chromatography (GCC), solvent systems as mobile phase (*n*-hexane, ethyl acetate), and Thin Layer Chromatography (TLC). The form of isolated compound was yellow needle crystals. The result of 1D-NMR (^{1}H and ^{13}C-NMR) data showed that the isolated compound was usnic acid. Antibacterial bioactivity testing was conducted by using the paper disk diffusion method indicated that the usnic acid compound was actively inhibited the growth of *E. coli* (ATCC35218) and *S. aureus* (ATCC25923) at the concentrations of 500mg/mL and 1000mg/mL with inhibition zone between 12mm and 17mm. In addition, *S. typhi* (YCTC) was only inhibited at the concentration of 1000mg/mL with inhibition zone of 14mm.

Keywords: Isolation, Lichen, *Usnea longissima* Ach., antibacterial, usnic acid

INTRODUCTION

In the last decade, traditional remedy has gained popularity and attracted people attention for its relatively small side effect on health (Shrestha *et al*., 2013; Shivanna *et al*., 2015; Shukla *et al*., 2015). The rising utilization of plants as medicine has begun since many years ago; it is proven by the publications of several books on medicinal plants and their research-based advantages (Huneck *et al*., 1996). Based on the findings of some researches, medicinal plants contain bioactive substance which can be developed/cultivated commercially (Thippeswamy *et al*., 2011).

Based on the record of WHO (World Health Organization), almost 80% of world population use plants as medicine (WHO, 2007). Indonesia is one of the tropical countries and it has the greatest biodiversity in the world following Brazil (Jenkins, 2003). There are more than 1100 kinds of plant containing secondary metabolite that are potential to be processed into foodstuff, cosmetics, and medicines, however, only 750 species which have been identified medicinal (Choudary *et al*., 2005; Maulidiyah *et al*., 2011).

People process many plants into medicine since they contain active substance that can cure many diseases (Pavithra *et al*., 2013). Some infectious diseases are triggered by bacteria, e.g. *Escherichia coli* is pathogen when its number inside gastronomic track or outside the intestine increases since it will produce enterotoxin which causes diarrhea (Odonkor *et al*. 2013). *Sthapylococcus aureus* frequently infects human and creates boil, pimples, impetigo, wound infection, and even meningitis in severe infection (Adejuwon *et al*., 2010). In addition to those two bacteria, *Salmonella typhi* also infects human or animal when it goes into the mouth. This bacterium is usually transmitted from animals and animal product to human. The transmitted bacterium then leads to typoid fever (*Thypoid fever*) (Chart *et al*., 2007). Typoid fever is an acute infectious disease that harms gastronomic tracks. The symptoms include high fever that lasts more than 7 days, gastronomic disorders, and/or loss of consciousness (WHO, 2003).

In Indonesia, alternative production of natural antibiotics from lower plants which contain secondary metabolite substance and biological activity is still limited. One kind of lower plants is lichen. It is rarely studied whereas, as a matter of fact, lichen has been used as medicine since middle century by a lot of physicians (Rauf *et al*., 2011; Xu M. *et al*., 2016). A review reported that secondary metabolites of lichen exhibit activities as antibiotics, antibacterial, anti-inflammation, analgesic, antipyretic, anti-proliferation, anti-genotoxin, antioxidant, and cytotoxic effect (Agar *et al*., 2011; Vivek *et al*., 2014). Furthermore, research findings reported that lichen of *U. blepharea* Motyka produces secondary metabolite substances in the forms of Eumitrin A_1 and (-)-usnic acid which exhibit very active response towards a toxicity test of Murine Leukemic P388 cell by 4.5µg/mL and 5.7µg/mL, respectively. It makes correlation with other bioactivity like anti-microbe possible (Maulidiyah *et al*., 2015a; 2015b).

Usnea longissima, a kind of lichen, is widely examined because it is expected to have more active secondary metabolite substance (Kocer *et al*., 2014; Prateeksha *et al*., 2016). *Usnea longissima* is in the family of Parmeliaceae and it lives by attaching itself to pine trees

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(Maulidiah *et al*., 2016). It is 15-35cm long or even longer; *Usnea longissima* is yellowish-green in color. In medical science, *Usnea longissima* has analgesic, antidote, astringent, and cardiotonic features as well as stands as a stomach medicine (He *et al*., 2012). It also stands as an agent to cure wounds, inflammation, cough, fever, detoxification, and increases lactation in women (Atalay *et al*., 2011; Sun *et al*., 2016). Many studies reported that environmental factor of the place where it grows can affect its secondary metabolite content (Latkowska *et al*., 2015; Mafole *et al*., 2016). Thus, this study reviewed the bioactivity of lichen, *U. longissima*, originating from Indonesia that has not been exposed. This study aimed at identifying the secondary metabolite substance of *U. longissima* and its bioactivity as anti-bacterial.

MATERIALS AND METHODS

Isolation

Lichen *U. longissima* Ach. was originally obtained from the mountain of Enrekang, South Sulawesi, Indonesia. The lichen *U. longissima* Ach. was identified by Department of Biology, Universitas Halu Oleo. Fine powder of lichen *U. longissima* Ach. was weighted to 710 g and maceration with 5.5L of methanol for 3×24 hours. Macerate was separated using a rotary vacuum evaporator until green extract produced. Subsequently, the partition process started by adding methanol extract into a separate funnel and partitioned with *n*-hexane by shaking and let stay till it has separated (polar and non-polar). The results of each partition were combined and made dense until extract produced.

Separation and purification

The extract of partition was tested using TLC to define the best ratio of eluent was used in column chromatography. The eluent used to separate the extract is mixture of *n*-hexane: ethyl acetate with the ratios of 10:0, 9:1, 8:2, to 0:10. The analysis of separation results was conducted by using UV light and stain reagent, i.e. Cerium Sulfate (Ce $(SO_4)_2$).

The 20g silica gel G60 adding into GCC, while 2g of sample had been dissolved with *n*-hexane and 4g silica gel G60 saved into the upper part of GCC. This was mixed thoroughly and stirred to dry (impregnation). After that, the elution process was conducted by following the order of eluent listed in table 1. Elusion process was applied by pouring non-polar eluent into a column. Then, after going through the column and eluent came out stored in a container. This process was conducted continuously with the gradual increase of eluent polarity to the most polar eluent.

All fractions produced within GCC were tested by using TLC plate in a chamber which contained proper eluent. Then, Rf (Retardation Factor) value of each fraction was calculated. The TLC results of each fraction were analyzed using UV light and stain reagent of Cerium (IV) sulfate ($Ce(SO_4)_2$). The same R_f value were combined and made dense.

A substance was considered pure was examined to identify its purification by using TLC with the three-solvent system. The results of TLC were observed under UV light and sprayed with stain reagent of Ce $(SO_4)_2$. If stain on the TLC plate remained single spot, the substance could be considered isolated. The last step, the structure of isolate was determined by using IR and 1D-NMR (^{1}H-NMR and ^{13}C-NMR) spectrophotometer. The resulted data were interpreted by comparing the data to other literature which could help the map of the structure of isolated.

Antibacterial bioactivity testing using paper disc diffusion method

Production and sterilization of media

Bacteria were rejuvenated in Nutrient Broth (NB) media. NB media was made by dissolving all compositions of NA (excluding the agar) in the form of yeast extract, peptone, NaCl, and MgSO4 in 100mL of distilled water and incubated for 24 hours. NA antibacterial media testing was made by dissolving 20 grams of NA into 1000mL of distilled water in Erlenmeyer 1L. The solution of NA 20g/L in that Erlenmeyer was heated using hot plate to boil and dissolve completely. Then, it was sterilized in an autoclave at the temperature of 121oC with 1 atm of pressure for 15 minutes. Tested microorganism used was consisted of E. coli, S. aureus, and S. typhi. Each bacterium was rejuvenated by transferring 1 or 2 loopful of culture that was put in oblique agar media into ampoule bottle containing NB liquid media; then, it was incubated for 24 hours at the temperature of 37±2°C.

Antibacterial bioactivity testing

In general, antibacterial bioactivity testing of particular substance uses positive control and negative control/solvent as the parameter of the potency of the tested substance in inhibiting bacteria. Positive controls such as amoxicillin, chloramphenicol and so forth are substances which have already known for their active antibacterial properties. Antibacterial activity test using the diffusion method. This method was conducted by utilizing disc which was inserted with antibacterial and placed in solid media which had been inoculated with indicator bacteria. After being incubated for 30 minutes at the temperature of 37°C, there emerged saturated area around the disc and the inhibition diameter. This inhibition zone was measured using a ruler in four repetitions. All analyzes use Microsoft Office Excel 2013.

STATISTICAL ANALYSIS

Antibacterial activity test using the diffusion method. All analyzes use Microsoft Office Excel 2013.

RESULTS

Isolation of natural substance

The sample was macerated, extracted and partitioned by using *n*-hexane in separating funnel. Eluent system used was *n*-hexane: Ethyl acetate with various increasing ratios of polarity. It was used to make the substances separated based on their degrees of polarity. Chromatography of *n*-hexane fraction exhibited different spots for each eluent ratios used. Eluent system that produced good separation was the ratio of *n*-hexane: ethyl acetate of 8:2, thus this ratio was used as the parameter in fractionation of column chromatography. Column chromatography produced 58 fractions. Each fraction places in ampoule bottle underwent TLC using eluent ratio of *n*-hexane: ethyl acetate of 8:2. fig. 1 exhibits the chromatogram of GCC. Based on the pattern of nodes, the fractions with the same pattern of nodes and R_f were combined based on the assumption that those fractions have the same substances. The results of combination of column chromatography fractions were presented in table 2.

Identification of isolate structure

Isolate compound that had been produced was in the form of golden-yellow needle-like crystal which melting point ranging from 201-203°C. Identification of substance from isolation was managed using spectroscopy method with IR (fig. 2), ^{13}C-NMR spectrophotometer (fig. 3 and table 3) and ^{1}H-NMR spectrophotometer (fig. 4 and table 3). Base on data of the Comparison of 1H-NMR and 13C-NMR Isolates and Usnic Acid (table 4) show structures of isolated compound can be seen in fig. 5.

Anti-bacterial bioactivity testing

Two samples were used in antibacterial testing; i.e. *n*-hexane extract and isolate compound. *n*-hexane was used as negative control in antibacterial testing of *n*-hexane fraction, while chloroform solvent was used as negative control in antibacterial activity testing of isolates. The solvents applied to both samples were used as negative control, an indicator to identify the effect of solvents on the samples activities. When the samples and the solvents are active in inhibiting the growth of the bacteria, it still cannot be inferred that the samples inhibited the growth.

Table 1: Data of eluent composition in GCC

Eluent Number	Eluent (*n*-hexane : ethylacetate)	Volume of *n*-hexane	Volume of ethylacetate	Total volume of *n*-hexane (mL)
1	10:0	100	0	100
2	9:1	180	20	200
3	8:2	240	60	300
4	7:3	140	60	200
5	6:4	60	40	100
6	5:5	50	50	100
7	4:5	40	60	100
8	3:7	30	70	100
9	2:8	20	80	100
10	1:9	10	90	100
11	0:10	0	100	100

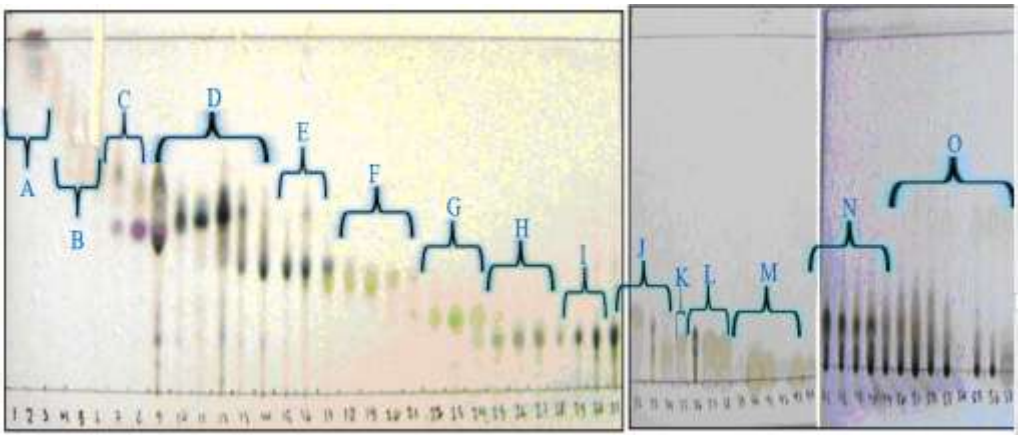


Fig. 1: GCC Chromatogram

Table 2: The results of combination of GCC Fractions

No.	Fractions	Combined Fraction	Weight (mg)	Color
1	1 - 3	A	90	Clear
2	4 - 6	B	120	Yellow
3	7 - 8	C	180	Sulfur
4	9 - 14	D	80	Green
5	15 - 17	E	260	Brownish green
6	18 - 21	F	80	Yellowish-brown
7	22 - 24	G	50	Brown
8	25 - 28	H	100	Yellow fade
9	29 - 31	I	50	Yellow
10	32 - 35	J	30	Yellow greenness
11	36	K	90	Green
12	37 - 39	L	40	Yellowish green
13	40 - 44	M	60	Yellow
14	45 - 50	N	130	Yellow fade
15	51 - 58	O	160	Yellow gold

On the other hand, when the samples are active and solvents are not active, it can be verified that the samples inhibit the growth of bacteria.

Bioactivity testing of n-hexane fraction as anti-bacterial
The results of this test (table 5) show that the extract of *n*-hexane was active towards the activity of *E. coli* at the concentrations of 500mg/mL and 1000mg/mL with inhibition zone of 11mm and 14mm, respectively. The extract was also active towards the activity of *S. typhi* at the concentration of 1000 mg/mL with inhibition zone of 12 mm.

Bioactivity testing of isolate substance as anti-bacterial
Isolate compound actively inhibited the growth of *E. coli* at the concentrations of 500mg/mL and 1000mg/mL with inhibition zone of 16mm and 17mm, respectively. It also actively inhibited the growth of *S. aureus* at the concentrations of 500mg/mL and 1000mg/mL with inhibition zone of 12mm. In addition, it actively inhibited the growth of *S. typhi* at the concentration of 1000mg/mL with inhibition zone of 14mm (table 6).

DISCUSSION

These combinations were tested using TLC to determine which fractions that were going to be pure further. The results of GCC fractions showed that A and O fractions have node stain which were nearly pure, characterized by crystal formation in the fractions. Based on the weight of crystals, O fraction gained more weight that made it possible to be pure further. O fraction exhibited the formation of crystal in the form of yellow needle that recrystallization was conducted towards this fraction. Recrystallization towards O fraction was administered by adding *n*-hexane solvent; the debris was dissolved, while the crystal was not. After that, TLC was administered using the various eluent system to examine purity. TLC test using various eluent systems produced residual material in the form of yellow crystal and exhibited a single node indicating that the isolate compound was pure. Then, purity testing was conducted by simultaneously dropping isolate compound and usnic acid (the standardized substance), isolated by Maulidiyah, on the same TLC plate (Maulidiyah *et al*., 2011). Isolate compound was also combined with usnic acid to ensure that the resulted spot remained single. The results of TLC showed that isolate compound had the same single spot and R_f as usnic acid that it was momentarily assumed that the successfully isolated compound was usnic acid.

Every functional group of isolate has different energy of stretching and bending as described by IR spectrum above (fig. 2). An adsorption band within the area of 3,089cm^{-1} is stretching vibration from saturate double bond or olefinic, while adsorption band within the area of 2,981cm^{-1} is the stretch vibration of asymmetrical C-H groups supported by adsorption band within the area of 1,452cm^{-1} that was the bending vibration of C-H group. Adsorption band within the area of 1,693cm^{-1} indicated the existence of a carbonyl group that was conjugated with C=C. Adsorption band within the area of 1,631cm^{-1} exhibited an adsorption band of aromatic ring. Based on the infrared spectrum data, it was estimated that isolate compound contained ketone group (C=O), hydroxyl group (OH), aromatic group and CH_3 group. The emergences of these functional groups were justified by the data of NMR spectrum.

^{13}C-NMR spectrum as presented in fig. 3 descried the number of carbon that constructs the structure of isolate compound. The result of this spectrum revealed 18 peaks of chemical shift (δ_c) indicating that isolate compound contained 18 carbon atoms. Three carbonyl atoms in δ_c scored the highest values; i.e.δ_c=198.2ppm, δ_c=200.4ppm, and δ_c =201.9ppm. The value of chemical shift was

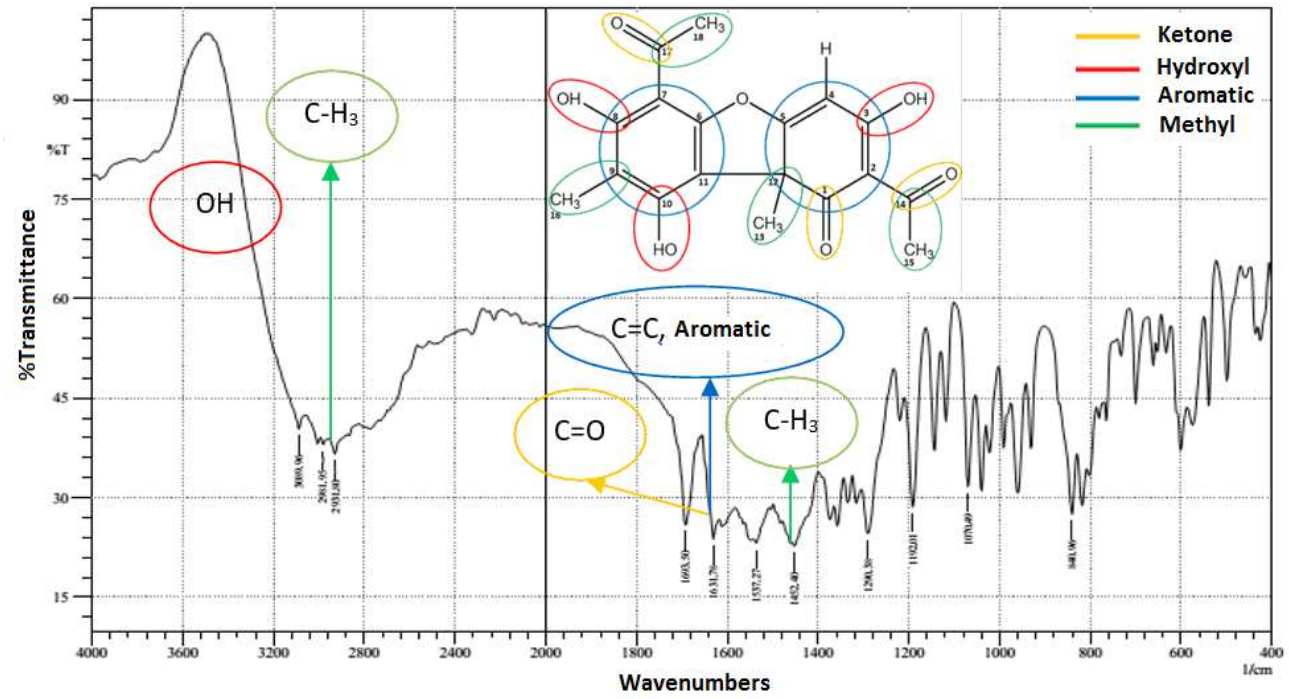


Fig. 2: IR spectrum of isolated compound

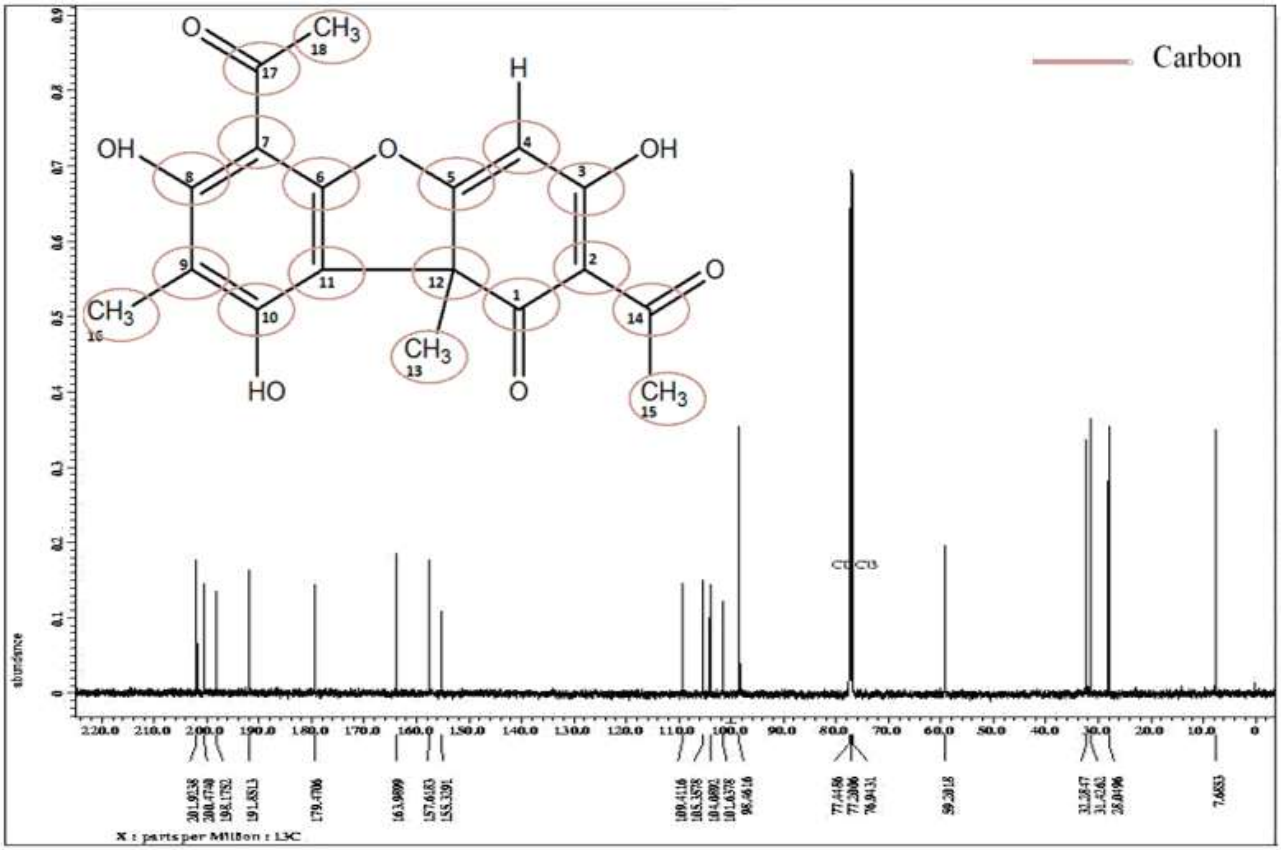


Fig.3: ^{13}C-NMR Spectrum of isolate compound

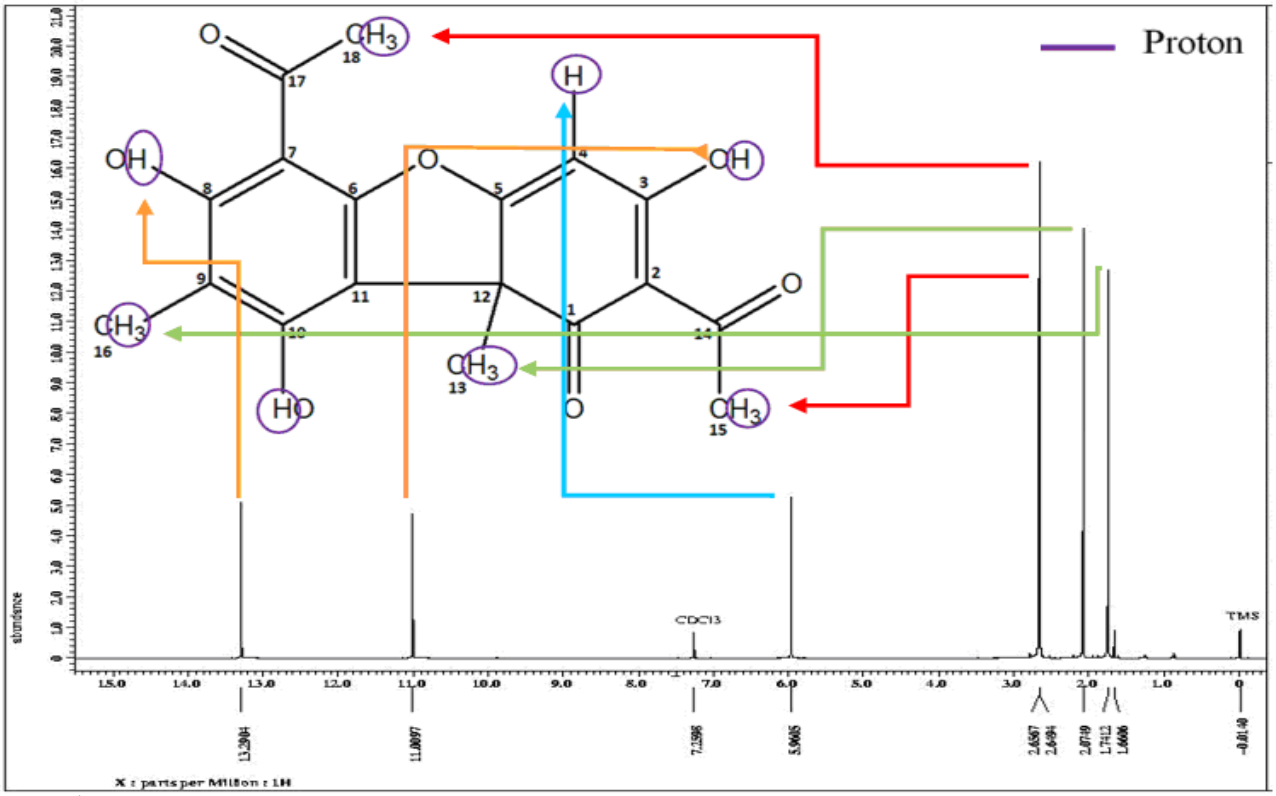


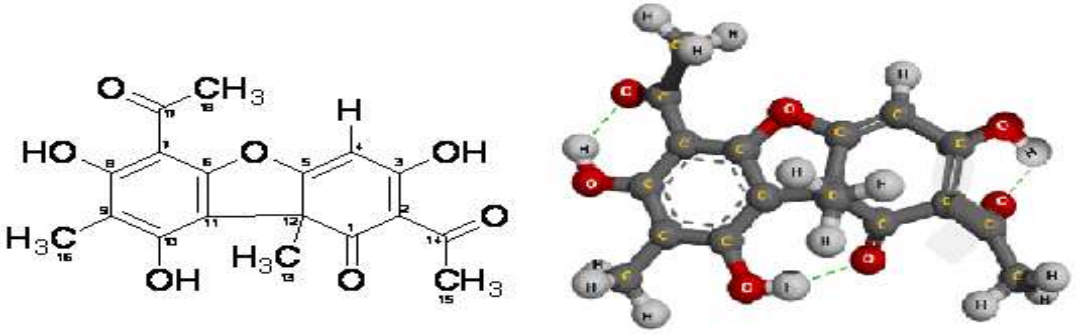
Fig. 4: ^{1}H-NMR spectrum of isolate compound

Table 3: The Data of Chemical shift of ^{1}H-NMR and ^{13}C-NMR of Isolate compound

C Position	^{1}H-NMR	^{13}C-NMR	Carbon type
1		198.20	C=O
2		179.47	Cq
3	11.01 (1H, *s*, HO-3)	155.33	Cq, COH
4	5.96 (1H, *s*, H-4)	98.46	CH
5		101.64	Cq, COH
6		98.46	Cq, C–O
7		109.41	Cq
8	13.29 (1H, *s*, CHO-8)	157.62	Cq, COH
9		104.09	Cq
10		163.99	Cq, COH
11		105.36	Cq
12		59.20	Cq
13	1.74 (3H, *s*, Me-13)	28.04	CH_3
14		200.47	C=O
15	2.66 (3H, *s*, Me-15)	32.28	CH_3
16	2.08 (3H, *s*, Me-16)	7.68	CH_3
17		201.92	C=O
18	2.65 (3H, *s*, Me-18)	31.43	CH_3

Where : The process of determining the number of double bonds or the rings of isolate compound was administered by using DBE (Double Bound Equivalence) with the formula of F = X – ½ Y + ½ Z + 1, F = 18 – ½ (16) + ½ (0) + 1 = 11

F = the number of rings or double bonds
X = the number of tetravalent atoms
Y = the number of monovalent atoms (H, F, B, Cl)
Z = the number of trivalent atoms (N, P)



(1D Structure of Isolate compound) (3D Structure of Isolate compound)

Fig. 5: Structures of isolated compound

Table 4: Data of the Comparison of ^{1}H-NMR and ^{13}C-NMR Isolates and Usnic Acid

No. C	δ_C Isolate (ppm)	δ_C[a] Usnic Acid (ppm)	Position H	δ_H Isolate (ppm)	δ_H[b] Usnic Acid (ppm)
1	198.2	198.2	HO-3	11.01 (1H, *s*)	11.03 (*s*)
2	179.4	179.4	H-4	5.96 (1H, *s*)	5.92 (*s*)
3	155.3	155.1	HO-8	13.29 (1H,s)	13.31 (*s*)
4	98.4	98.3	H-13	1.74 (1H, *s*)	1.75 (*s*)
5	101.6	101.7	H-15	2.66 (1H, *s*)	2.66 (*s*)
6	98.4	99.8	H-16	2.07 (1H, *s*)	2.10 (*s*)
7	109.4	109.5	H-18	2.65 (1H, *s*)	2.63 (*s*)
8	157.6	157.6			
9	104.0	104.2			
10	163.9	164.0			
11	105.3	105.4			
12	59.2	59.2			
13	28.0	27.4			
14	200.4	200.1			
15	32.2	32.0			
16	7.6	7.7			
17	201.9	201.3			
18	31.4	30.9			

Table 5: The Results of Anti-Bacterial Testing of *n*-hexane Fraction

No	Bacterium's	Diameters of clear zone (mm)					
		Fraction of *n*-Hexane (mg/mL)				*n*-Hexane	Ampicillin (1000mg/mL)
		100	250	500	1000		
1	*E.coli* ATCC 35218	6	6	11	14	6	21
2	*S.auerus* ATCC 25923	6	6	6	7	6	28
3	*S. typhi* YCTC	6	6	6	12	6	17

Table 6: The Results of Anti-Bacterial Test of Pure Isolate

No	Bacterium's	Diameters of clear zone (mm)					
		Isolate compound (mg/mL)				$CHCl_3$	Ampicillin (1000 mg/mL)
		100	250	500	1000		
1	*E.coli* ATCC 35218	9	10	16	17	6	17
2	*S.auerus* ATCC 25923	7	8	12	12	6	29
3	*S. typhi* YCTC	7	8	8	14	6	12

The diameter of Whatman disc = 6 mm

205ppm; chemical shift of δ_c of 198.2ppm was caused by carbonyl group conjugated with double bonds that resulted in resonance (Maulidyah *et al*., 2015a). It was supported by the formation of IR adsorption band within

the wavelength of 1,693cm^{-1} which was less than the wavelength of normal carbonyl of 1,715cm^{-1}. Peak with quite great chemical shift was also identified within the chemical shift of δ_c of 157.6ppm, δ_c of 163.9ppm, and δ_c of 191.8ppm; these indicated the configuration of carbon atoms which were bound to hydroxyl group, in which that hydroxyl group was directly bound to aromatic group. Besides, chemical shift of δ_c of 7.6ppm and δ_c of 28.0ppm with the least δ_c peak and high peak intensity indicated the structure of carbon methyl atom. Carbon methyl atom in isolate was also found within the chemical shift of δ_c of 31.4ppm and δ_c of 32.3ppm which were bound to carbonyl group.

^{1}H-NMR data indicated that isolate compound had 16 protons. There were 2 methyl groups observed in chemical shift of δ_H =1.74 (s) and 2.08 (s) ppm. However, chemical shift of δ_H of 2.66ppm and 2.65ppm indicated the structures of two methyl groups bound to carbonyl group. Besides, the spectrum of chemical shift of δ_H of 13.29ppm and 11.01ppm indicated the structures of two hydroxyl groups which formed hydrogen bond with carbonyl group.

Based on the data of ^{13}C-NMR, it was estimated that the molecular formula of isolated compound was $C_{18}H_{16}O_7$ with DBE including 11 atoms from carbonyl (C=O), double bonds from aromatic group, and 3 rings from isolate compound. Based on the results of IR and NMR (1H-NMR and 13C-NMR) measurements of isolated compound, further justification was conducted by comparing similar data from literature presented in table 4. Those data indicated the similarity of ^{1}H-NMR and ^{13}C-NMR spectrums of isolated compound and usnic acid. The data strengthen the hypothesis that the substance which had been successfully isolated from *n*-hexane of lichen *U. longissima* was usnic acid. Based on the research findings reported by Maulidiyah *et al*. that lichen of *Usnea sp.* produces secondary metabolite substances in the forms of (-)-usnic acid. It makes possible correlation with other bioactivity (Maulidiyah *et al*., 2016; Maulidiyah *et al*., 2015b)

Ampicillin was used as positive control, a benchmark indicator towards the samples. If the diameters of clear zone of samples are greater than ampicillin's, it can be said that the samples are very active antibacterial. In addition, when the diameters of clear zones of the samples are less than ampicillin's, it can be stated that the samples are not effective antibacterial substances.

Based on the test results and the comparison of classification data of bacterial growth inhibition response presented in table 5, it can be stated that *n*-hexane extract has low inhibition response towards the growth of *E. coli* and *S. typhi*, and it does not actively respond the activity of *S. aureus*. These results indicate that *n*-hexane extract is not too efficient to be used as antibacterial substance.

Usnic acid is bacteriostatic in low concentration and bactericidal in high concentration. If the concentration of usnic acid is getting higher, the diameter of clear zone will be greater too. Isolate compound in the form of cyclical ring has carbonyl groups and oxygen bridge (R-O-R) which are active because they can act as DNA intercalator attached to 2 units of base pairs of DNA and interact with DNA through Van der Walls bond that may harm the double helix and prevent the copy of DNA.

Based on the results of antibacterial testing and the comparison of classification data of inhibition responses toward bacteria growth represented in Table 6, isolate compound has moderate inhibition response towards the growth of *E. coli*. However, its response towards the growth of *S. aureus* and *S. typhi* is relatively low. These results indicate that isolate compound is relatively efficient to be used as antibacterial agent, especially towards *E. coli*.

CONCLUSION

The secondary metabolite substance isolated from *U. longissima* Ach. was usnic acid compound with molecular formula $C_{18}H_{16}O_7$. Antibacterial bioactivity test showed that the usnic acid compound was actively inhibited the growth of *E. coli* (ATCC35218) and *S. aureus* (ATCC25923) at the concentrations of 500mg/mL and 1000mg/mL with inhibition zone between 12mm and 17mm. In addition, *S. typhi* (YCTC) was only inhibited at the concentration of 1000mg/mL with inhibition zone of 14mm.

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