

Secondary Metabolites from *Usnea* sp. and an Evaluation of Their Cytotoxic and Antibacterial Activities

Dewi Meliati Agustini¹  , Riga Riga^{*2}  , Elvira Hermawati³  , Budi Saputra¹  

¹Department of Chemistry, Faculty of Science and Technology, Universitas Jenderal Achmad Yani, Cimahi, Indonesia.

²Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Padang, Indonesia.

³Department of Chemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung, Indonesia.

*Corresponding Author.

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Abstract

The genus *Usnea* was reported to synthesize the bioactive secondary metabolites, including cytotoxic and antibacterial activities. To continue our study on bioactive compounds from *Usnea* sp, we studied secondary metabolites and their bioactivities of *Usnea* sp. The aim of this study was to determine the chemical constituents of lichen *Usnea* sp. Methanol extract of *Usnea* sp. was prepared by solid liquid extraction followed by fractionation and purification of the crude extract by several chromatographic techniques using *n*-hexane and ethyl acetate as solvents to obtain two pure compounds (**1** and **2**). The structures of both secondary metabolites were analyzed by 1D and 2D (one and two-dimensional) NMR spectroscopy and identified as 2,6-dihydroxy-4-methylbenzoic acid (**1**) and (-)-placodiolic acid (**2**). Cytotoxic activity of compounds against MCF-7 cells was evaluated using the MTT assay (IC₅₀ = 554.82 and 5.10 μM, respectively). Both compounds were also assayed for their antibacterial activity using disc diffusion method. Inhibition zones of compound **1** against *S. aureus*, *E. coli*, and *S. pyogenes* were 6.37 ± 0.21, 7.57 ± 0.15, and 5.43 ± 0.12 mm, respectively. In addition, compound **2** inhibited the growth of *S. aureus*, *E. coli*, and *S. pyogenes* bacteria (Inhibition zones = 10.43 ± 0.25, 11.63 ± 0.21, and 10.63 ± 0.21). The result of biological activities demonstrated that **2** exhibited stronger cytotoxic activity against MCF-7 cells as well as antibacterial activity than compound **1**. Based on the theses results, compound **2** could be a promising candidate of antibiotics in the near future.

Keywords: Antibacterial, cytotoxicity, MCF-7 cells, secondary metabolites, *Usnea* sp.

Introduction

Usnea is a lichen genus in family Parmeliaceae growing like leafless mini-shrubs or tassels anchored on bark or twigs. Traditionally, *Usnea* sp. has been used as medicine including antimicrobial and anticancer agents^{1,2}. Most of the lichen *Usnea* sp. scattered in the tropics and others in the sub-tropics. The morphology of the *Usnea* is a branched thread-like tallus shape, greenish-white color, and vertical growth^{3,4}.

Previous phytochemical study reported that *Usnea* sp. produced benzofuran derivatives, depsides, depsidones, terpenoids, steroids, and phenolic compounds⁵⁻⁷. Some of the reported compounds exhibited several activities including anti-inflammatory, antibacterial, antifungal as well as cytotoxic activity⁸⁻¹⁰. Two isolated compounds namely usnic acid and diffractic acid from *Usnea* sp. exhibited antioxidant activity with IC₅₀ values of 7.67 and 18.51 μg/ml, respectively¹¹. In addition,

usnic acid obtained from *U. longissimi* Ach. inhibited the growth of *E. coli* (ATCC35218) and *S. aureus* (ATCC25923) with inhibition zones between 12mm and 17mm¹². Diffraction acid isolated from *U. blepharea* Motyka actively inhibited (very strongly) the growth of *S. aureus* at concentrations of 750 and 1,000 ppm with inhibition zones of 14.27 and 17.25 mm, respectively. In addition, *E. coli* was also actively inhibited (strongly) at concentrations of 750 and 1,000 ppm at 11.00 and 12.75 mm, respectively¹³.

Furthermore, (-)-usnic acid from *Usnea* sp. showed cytotoxicity against murine leukemia P388 cells with IC₅₀ 5.738 ± 0.61 µg/mL¹⁴. Four new tetrahydroxanthone–chromanone heterodimers, usneaxanthenes E–H as well as eleven known compounds were isolated from lichen *Usnea*

aciculifera Vain and showed potent cytotoxicity against HCT116 colon cancer with IC₅₀ values from 3.37 to 4.53 µM¹⁵. The previous data of cytotoxic and antibacterial activities by isolated compounds from *Usnea* indicated that *Usnea* is source of cytotoxic and antibacterial compounds.

In continuing study for searching bioactive compounds from lichen *Usnea* sp., we have studied the chemical constituents on lichen *Usnea* sp. In this study, two secondary metabolites as in Fig 1 have been isolated from the methanol extract of *Usnea* sp. Spectroscopic analysis was used to determine the structures of isolated compounds. Cytotoxic and antibacterial activities of both compounds were also assayed. Interestingly, compound **1** is firstly reported from *Usnea* sp. herein.

Materials and Methods

Instrumentation

An Agilent Varian spectrometer (Agilent Technologies, United States) at 500MHz (¹H-), and 125MHz (¹³C-) using Me₄Si as reference and CD₃OD as solvent was used to obtain the NMR spectra. An Autopol IV polarimeter (Rudolph Research Analytical, United States) was used to determine the optical rotation. Silica gel plates (Silica gel 60 F254, 20 x 20 cm, Merck, Germany) was used for thin layer chromatography. Silica gel Kieselgel 60, 0.04-0.063 mm (Merck, Germany) was used to vacuum liquid chromatography (VLC) and column chromatography. UV detector (254 and 336 nm) was used to detect the TLC spots.

Lichen material

Lichen *Usnea* sp. was collected on December 2020 from Ciwidy, South Bandung, West Java, Indonesia, latitude: 7.0849° S, longitude: 107.4461° E, elevation: 1169 m.

Extraction and isolation

The dried Lichen *Usnea* sp. (2 kg) was extracted with methanol⁶ to obtain crude extract (30.8 g) which was tested by their cytotoxic activity. The crude extract (20 g) was fractionated using VLC technique (solvents: gradient system (*n*-hexane and ethyl acetate = 100:0 – 0:100))¹⁶ to give nine major fractions (A-I). Two largest mass fractions (H and I) were further fractionated. Fraction H (1.33 g) was further fractionated over silica gel vacuum liquid chromatography (solvents: with *n*-hexane and ethyl acetate (100:0 – 0:100)) to result 16 sub-fractions

(H.1-H.16). Sub-fractions H.15 and H.16 were subjected to silica gel radial chromatography (solvents: gradient system of *n*-hexane and ethyl acetate) obtaining compound **1** (1.2 mg).

Fraction I (1.98 g) was fractionated over silica gel vacuum liquid chromatography (solvents: gradient system of *n*-hexane and ethyl acetate) to give 25 sub-fractions (I.1-I.25). Sub-fractions I.9, I.10, and H.11 were subjected to silica gel radial chromatography (solvents: *n*-hexane and ethyl acetate (7:3)) obtaining compound **2** (12.8 mg).

Characteristic data of isolated compounds

2, 6-dihydroxy-4-methylbenzoic acid (**1**)

Yellow solid (1.2 mg). Soluble in acetone. R_f = 0.65 (*n*-hexane and ethyl acetate = 1:1). ¹H-NMR ((CD₃)₂CO at 500 MHz): δ 13.99 (s, 1H, -COOH), 11.46 (s, 2H, 2-OH dan 6-OH), 6.99 (s, 2H, H-3/H-5), and 2.79 (br, 3H, H-7). ¹³C-NMR ((CD₃)₂CO at 125 MHz): 172.3 (C10), 160.7 (C4/6), 146.3 (C5), 107.9 (C1/3), 98.9 (C2), and 21.6 (C9).

(-)-Placodiolic acid (**2**)

Yellow solid (1.2 mg). Soluble in acetone. R_f = 0.70 (*n*-hexane and ethyl acetate = 1:1). ¹H-NMR ((CD₃)₂CO at 500 MHz): δ 13.44 (s, 1H, 7-OH), 11.46 (s, 1H, 9-OH), 3.53 (s, 3H, H-16), 3.14 (s, 1H, 3-OH), 3.04 (s, 2H, H-4), 2.54 (s, 3H, H-15), 2.31 (s, 3H, H-12), 1.92 (s, 3H, H-13), and 1.50 (s, 3H, H-10). ¹³C-NMR ((CD₃)₂CO at 125 MHz): δ 202.0 (C-14), 198.9 (C-11), 191.8 (C-3), 163.5 (C-7), 163.0 (C-9), 161.2 (C-5), 157.9 (C-1), 114.1 (C-2), 111.8 (C-4a), 108.0 (C-9a), 105.6 (C-6), 101.8 (C-8), 59.9

(C-9b), 51.6 (C-16), 42.4 (C-4), 32.0 (C-12), 31.3 (C-15), 19.3 (C-10), and 7.5 (C-13).

In-vitro cytotoxic assay

Both secondary metabolites (**1** and **2**) were assayed for their cytotoxicity against MCF-7 breast cancer line using MTT method¹⁶⁻¹⁹ with modification. Cells of MCF-7 in 96-well plates with cell density of 3×10^4 cells/cm³ were incubated for 24 hours. Then, the compounds with several concentrations were added and incubated for 48 h. Every compound was dissolved in dimethyl sulfoxide (DMSO). Negative control wells received only DMSO. Then, 10 μ L MTT reagents were added into each well. After 4 h incubation, the MTT-stop solution containing SDS (Sodium Dodecyl Sulfate) were added. After 24 h, a microplate reader ($\lambda = 550$ nm) was used to determine the optical density.

Results and Discussion

Compound **1** was isolated as a yellow solid. Spectrum of ¹H-NMR in Table 1. of **1** showed signals for a tetrasubstituted benzene ring [$\delta_H = 6.98$ ppm (2H, brs), H-3/H-5] where both protons were symmetrical H atoms, a methyl proton ($\delta_H = 2.79$ ppm, 3H, s, H-7), and a COOH proton at $\delta_H = 13.99$ ppm (1H, s). The fragment of compound **1** based on the spectrum of ¹H-NMR indicated the compound **1** was the 1, 2, 4, 6-tetrasubstituted benzene where the substituents of it were two hydroxyl groups, a methyl, and a carboxylic group. The result of comparing this data with literature confirmed compound **1** as 2, 6-dihydroxy-4-methylbenzoic acid Fig 1.

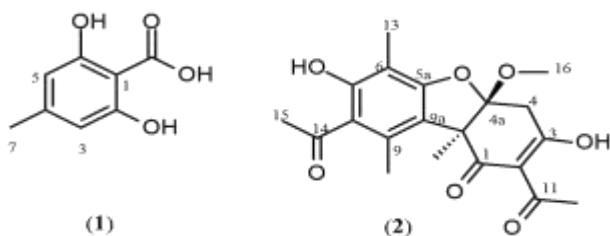


Figure 1. Structures of compounds 1-2 isolated from *Usnea* sp.

Several phenolic compounds which have similar structure with compound **1** have been isolated from various species of *Usnea*. *Usneaceratina B* which has a different position of the hydroxyl group with compound **1** was isolated from *Usnea ceratina*²². Furthermore, divaric acid and divaricatic acid with

Antibacterial assay

Two secondary metabolites were evaluated for their antibacterial activity with disc diffusion method following the reported procedure^{20, 21} with modification. *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pyogenes* were used for tested bacteria. Amoxicillin was used as a positive control. 15 ml of MHA media were poured into Petri dishes followed by inoculation of tested bacteria. Sterile paper disc (6 mm) was placed on the MHA media. Serial concentrations of isolated compounds were dropped on the paper disc and incubated for 24 hrs. The inhibition zone of isolated compounds was performed three times. Statically, the zone of inhibition was calculated and presented as mean \pm standard deviation²⁰.

propyl group at C-6 have been isolated from *Usnea barbata* 2017-KL-10²³. However, the chemical study of compound **1** from *Usnea* sp. first reported in this study. The isolation of compound **1** in this research completed the phytochemical profile of Lichen *Usnea* sp.

Table 1. ¹H-NMR data of compound 1.

Position	¹ H (δ_H (ppm), i, m, J)
1	-
2/6	-
3/5	6.98 (2H, s)
4	-
7	2.79 (3H, brs)
-OH	11.46 (2H, s)
-COOH	13.99 (1H, s)

Compound **2** was isolated as a powder (yellow). Spectrum of ¹H-NMR of **2** displayed three hydroxyl signals [$\delta_H = 13.44$ ppm (1H, s), $\delta_H = 11.46$ ppm (1H, s), and $\delta_H = 3.14$ ppm (1H, s)], a methoxy group at $\delta_H = 3.53$ ppm (3H, s), four methyls [$\delta_H = 2.54$ ppm (3H, s), $\delta_H = 2.31$ ppm (3H, s), $\delta_H = 1.92$ ppm (3H, s), and $\delta_H = 1.50$ ppm (3H, s)] and a methylene signal at $\delta_H = 3.04$ ppm (2H, s). Spectra of 1D NMR (¹H- and ¹³C-) as well as HSQC of compound **2** showed signals for three carbonyl carbons (δ_C 202.0, 198.9, and 191.8 ppm), four *sp*² oxy quaternary carbons (δ_C 163.5, 163.0, 161.2, and 157.9 ppm), four *sp*² oxy carbons (δ_C 114.1, 108.0, 105.6, and 101.8 ppm), a *sp*³ oxy quaternary carbon at δ_C 111.8 ppm, a *sp*³

quaternary carbon at δ_C 59.9 ppm, a methoxy carbon (δ_C 51.6 ppm), a sp^3 methylene at δ_C 42.4 ppm, and four methyl carbons (δ_C 32.0, 31.3, 19.3, and 7.5 ppm). Data of 1D-NMR of compound **2** are displayed in Table 2.

Chemical structure of **2** was confirmed by correlation of long correlation of H to C in HMBC spectrum. Methyl group (δ_H 2.54, H-15) was correlated with C-8 (δ_C 101.8), C-14 (δ_C 202.0) indicating the methyl as a part of alkyl group of ester connecting to benzene ring at C-8. Furthermore, a long-range correlation from methyl proton (δ_H 1.50, H-10) to quaternary carbon (C-4a, δ_C 111.8; C-9a, δ_C 108.0 and C-9b, δ_C 59.9) showed C10-C9a connected with a benzene ring and cyclic structure. The result of comparing the NMR data with the literatures^{24, 25} confirmed compound **2** as (-)-placodiolic acid Fig 1. Compound **2** has been reported from the Fungus *Mycosphaerella* sp., isolated from marine sediment. This study also indicated that compound **2** showed antibacterial activity against *Escherichia coli* ATCC 11775, *Klebsiella pneumonia* ATCC 4352, and *Staphylococcus aureus* ATCC 6538²⁴. Furthermore, compound **2** was isolated from *Leprocaulon microscopicum* acetone extract. This study was also revised for ¹³C- assignments of compound **2**²⁶.

Table 2. ¹H and ¹³C-NMR data of compound 2.

Position	¹ H (δ_H (ppm), i, m, J)	¹³ C (δ_C , ppm)
1	-	157.9 (=C _q)
2	-	114.1 (=C _q)
3	-	191.8 (CO)
4	3.04 (2H, s)	42.4 (CH ₂)
4a	-	111.8 (-C _q)
5	-	161.2 (=C _q)
6	-	105.6 (=C _q)
7	-	163.5 (=C _q)
8	-	101.8 (=C _q)
9	-	163.0 (=C _q)
9a	-	108.0 (=C _q)
9b	-	59.9 (-C _q)
10	1.50 (3H, s)	19.3 (CH ₃)
11	-	198.9 (CO)
12	2.31 (3H, s)	32.0 (CH ₃)
13	1.92 (3H, s)	7.5 (CH ₃)
14	-	202.0 (CO)
15	2.54 (3H, s)	31.3 (CH ₃)
16	3.53 (3H, s)	51.6 (-OCH ₃)
3-OH	3.14 (1H, s)	-
7-OH	13.44 (1H, s)	-
9-OH	11.46 (1H, s)	-

The pure compounds (**1–2**) was examined for their cytotoxicity against MCF-7 cells with cisplatin as a positive control. The categories of cytotoxic values are active with $IC_{50} < 10.00 \mu M$, moderate with $IC_{50} 10.00-40.00 \mu M$, and inactive with $IC_{50} > 40.00 \mu M$ ²⁷. Based on them, compound **1** was inactive with IC_{50} value of 554.82 μM . In the contrast, compound **2** was active against MCF-7 cells (active, IC_{50} value of 5.10 μM). The strong activity of compound **2** indicated that compound **2** might be useful as anticancer agents.

Both compounds were also evaluated for their antibacterial activity against *S. aureus*, *E. coli*, and *S. pyogenes* using disc diffusion method. Amoxicillin was used as a positive control and DMSO was used as a negative control. Compound **2** showed the greatest inhibition zone at a concentration of 5% against all tested bacteria. Data of biological activities displayed at Table 3.

Table 3. The IC_{50} and antibacterial activity (ZI, mm) of compounds 1 and 2.

Compound	Cytotoxicity (IC_{50} , μM)	Inhibition Zones (mm)		
		<i>S. aureus</i>	<i>E. coli</i>	<i>S. pyogenes</i>
1	554.82	6.37 ± 0.21	7.57 ± 0.15	5.43 ± 0.12
2	5.10	10.43 ± 0.25	11.6 ± 0.21	10.63 ± 0.21
DMSO (-)	-	0	0	0
Amoxicillin (+)	-	15.57 ± 0.15	16.3 ± 0.21	15.43 ± 0.25

Conclusion

In the present research, phytochemical study of two secondary metabolites from methanol extract of *Usnea* sp. was described. Both compounds were 2,6-dihydroxy-4-methylbenzoic acid (**1**) and (-)-placodiolic acid (**2**). Interestingly, compound **1** is isolated from *Usnea* sp. for the first time. Compound **2** exhibited significant cytotoxicity (IC₅₀ 5.10 μM) against MCF-7 cells and showed antibacterial

activity against three tested bacteria at concentration of 5%. The distinguished result of IC₅₀ and inhibition zones by both compounds indicated that compound **2** more potent than compound **1** as cytotoxic and antibacterial activities. In the future, compound **2** could be studied as a promising candidate of anticancer and antibiotic.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.

- Ethical Clearance: The project was approved by the local ethical committee at University of Universitas Negeri Padang, Padang, Indonesia.
- No animal studies are present in the manuscript.
- No potentially identified images or data are present in the manuscript.

Authors' Contribution Statement

D.M.A. planned the research work, contributed to the writing of the paper and data collection. R.R. contributed to the writing, data analysis of the paper and publishing the paper as correspondence author.

E.H. planned the research work and performed the experiments. B.S. contributed to the writing of the paper and data collection.

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المستقلبات الثانوية من اوسينا اس بي وتقييم أنشطتها السامة للخلايا والمضادة للبكتيريا

ديوي ميلياتي أوغستيني¹، ريجا ريجا²، الفيرا هيرماوتي³، بودي سابوترا¹

¹ قسم الكيمياء، كلية العلوم والتكنولوجيا، جامعة جيندرال أحمد ياني، سيماهي، إندونيسيا.

² قسم الكيمياء، كلية الرياضيات والعلوم الطبيعية، جامعة نيجيري بادانج، بادانج، إندونيسيا.

³ قسم الكيمياء، كلية الرياضيات والعلوم الطبيعية، المعهد التكنولوجي باندونج، باندونج، إندونيسيا.

الخلاصة

من المعروف عن جنس اوسينا بأنه يقوم بتجميع المستقلبات الثانوية النشطة بيولوجيًا، بما في ذلك الأنشطة السامة للخلايا والمضادة للبكتيريا. لمواصلة دراستنا حول المركبات النشطة بيولوجيًا من اوسينا اس بي، قمنا بدراسة المستقلبات الثانوية ونشاطها الحيوي في *Usnea sp.* كان الهدف من هذه الدراسة هو تحديد المكونات الكيميائية لنبات الحزاز اوسينا اس بي مستخلص الميثانول من اوسينا اس بي تم تحضيره عن طريق استخلاص السائل الصلب متبوعاً بتجزئة وتنقية المستخلص الخام بواسطة عدة تقنيات كروماتوغرافية باستخدام الهكسان و خلات الإيثيل كمذيبات للحصول على مركبين نقيين (1 و 2). تم تحليل هياكل كل من المستقلبات الثانوية بواسطة التحليل الطيفي للرنين المغناطيسي النووي (D1 و D2) أحادي وثنائي الأبعاد) وتم تحديدها على أنها حمض 6،2-ثنائي هيدروكسي -4-ميثيل بنزويك (1) و(-)-حمض بلاكوديوليك (2). تم تقييم النشاط السمي للخلايا للمركبات ضد خلايا MCF-7 باستخدام اختبار $IC_{50} = 554.82$ و $MTT = 5.10$ ميكرومتر، على التوالي). تم أيضًا تقييم كلا المركبين لنشاطهما المضاد للبكتيريا باستخدام طريقة الانتشار القرصي. كانت مناطق تثبيط المركب 1 ضد المكورات العنقودية الذهبية والإشريكية القولونية والبكتيريا المقيحة 0.21 ± 6.37 و 0.15 ± 7.57 و 0.12 ± 5.43 ملم على التوالي. بالإضافة إلى ذلك، يمنع المركب 2 نمو بكتيريا *S. aureus* و *E. coli* و *S. pyogenes* مناطق التثبيط = 0.25 ± 10.43 ، 0.21 ± 11.63 ، و 0.21 ± 10.63). أظهرت نتيجة الأنشطة البيولوجية أن 2 أظهر نشاطًا سامًا للخلايا أقوى ضد خلايا MCF-7 بالإضافة إلى نشاط مضاد للبكتيريا مقارنة بالمركب 1. وبناءً على نتائج الأطروحات، يمكن أن يكون المركب 2 مرشحًا واعدًا للمضادات الحيوية في المستقبل القريب.

الكلمات المفتاحية: مضاد للجراثيم، السمية الخلوية، خلايا MCF-7، المستقلبات الثانوية، اوسينا اس بي.