Potential of Usnic Acid Compound from Lichen Genus *Usnea* sp. as Antidiabetic Agents

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Abstract: Lichen *Usnea* sp. is potential as a new natural medicine. This study report isolation of secondary metabolites from lichen *Usnea* sp. and α-glucosidase inhibitory, which is potential as an antidiabetic agent. Lichen powder was macerated using methanol, separated using column chromatography gravity and thin-layer chromatography. The crystalline was isolated and purified by the recrystallization process for obtaining pure compound. The isolated compound was determined using FTIR and NMR spectroscopy (¹H and ¹³C). The results showed that the isolated compound was yellow needle crystals. Based on the spectra data interpretation, it was obtained usnic acid compound with the molecular formula of C₉H₅O₅. The α-glucosidase inhibitory activity test showed that the usnic acid had activity in inhibiting the α-glucosidase enzyme with an IC₅₀ value of 106.78 µg/mL. The usnic acid from *Usnea* sp. has a very good impact on the source of natural compounds as an antidiabetic drug in the future.

Key words: lichen, *Usnea* sp., antidiabetic, α-glucosidase, usnic acid

1 Introduction

The research on natural medicine compounds has become an existence in the search for new medicinal raw materials. With a tropical climate, Indonesia has great potential as an exploration area for natural compounds, such as lichen. Solárová et al.¹ have reported that lichen has medicinal potential due to the production of its unique chemical compounds not found in other plant organisms. What is more, lichen is easy to find on trees, rocks, and soil²⁻⁴. The lichen group that is most common in several countries is the lichen genus *Usnea*. Lichen *Usnea* has diverse species, such as *U. longissima*, *U. articulata*, *U. sternaii*, *U. vrieseana*, *U. blepharea*, *U. comosa*, *U. flexuosa*, *U. bayleyi*, *U. javanica*, and *U. dasypoga⁵*. Isolation of secondary metabolites from lichen *Usnea* has been reported, such as atranorin⁶, thamnolic acid⁷, diphptic acid, and usnic acid⁸. *Usnea* sp. is one of the genus of lichen that produces secondary metabolites, which are known to have many pharmacological activities as antibacterial⁹, anti-inflammatory¹⁰, antiproliferative¹¹, antioxidative¹², and α-glucosidase inhibitory¹³. There are still few reports of secondary metabolites from lichens *Usnea* sp. as an antidiabetic agent. Based on data from the International Diabetes Federation¹⁴, the prevalence of global diabetes is expected to increase by 552 million by 2030. Data from the World Health Organization (WHO)¹⁵ also reports that diabetes mellitus is the seventh-largest cause of death. One of the essential therapeutic methods for reducing plasma glucose levels is to inhibit α-glucosidase activity¹⁶⁻²⁰. Several previous studies have reported the potential of lichen exploration as a good antidiabetic agent.

The α-amylase inhibition test which has shown a positive potency there are 22 types of lichen, such as *Ramalina conduplicans*, *Usnea sinensis*, *Everniastrum cirrhatum*, *R. hossei*, *Parmotrema pseudotinctorum*, *Flavoparmelia caperata*, *Physcia aipolia*, *Heterodermia leucomela*, *P. reticulatum*, *P. tinctorum*, *U. articulata*, *R. pollinaria*, *R. hycana*, *R. sinensis*, *Herpothallon sp.*, *Cladonia rei*, *P. chinense*, *Punctelia subrubracta*, *Punctelia borreri*, *Hyperphyscia adglutinata*, and *Peltigera praetextata²¹⁻²⁶*. However, α-glucosidase inhibitory activity showed only five types of lichen, namely *Ramalina*

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celastri, R. nervulosa\textsuperscript{25}, Caloplaca biatorina\textsuperscript{26}, Cladonia sp.\textsuperscript{27,28} and T. flavicans\textsuperscript{29}.

This study’s novelty explores secondary metabolites from the lichen genus Usnea sp. as an antidiabetic agent. Based on our previous study, the lichen Usnea sp. contains secondary metabolites such as 2',3'-dihydroxy-7-allyl-4-one\textsuperscript{30}, 3'-[1'-(2',3'-dihydroxyphenyl)-propyl]-7-hydroxy-chroman-4-one\textsuperscript{31}, (5E,6E) 5-ethylidene-7-formyl-6,7-dihydroxytetratetra-6-enoate\textsuperscript{32}, eumitrin A1\textsuperscript{33}, atranorin\textsuperscript{6}, and diffractaic acid\textsuperscript{60}. Isolation, structural elucidation, and inhibition test of the α-glucosidase enzyme of a usnic acid compound from lichen genus Usnea sp. will be discussed in detail.

2 Methods

2.1 Chemicals

Lichen Usnea sp. was obtained from Lompobatang Village, Tinggi Moncong District, Gowa Regency, South Sulawesi, Indonesia. Methanol, acetone, ethyl acetate, n-hexane, dichloromethane, chloroform, and distilled water were purchased from Sigma-Aldrich, USA. Sulfuric acid (10%), dihydrogen phosphate, disodium hydrogen phosphate, silica gel (0.063-0.200 mm), p-nitrophenyl-α-D-glucopyranoside, α-glucosidase enzymes, sodium carbonate (0.2 M), and dimethyl sulfoxide were purchased from Merck, Germany.

2.2 Extraction of lichen Usnea sp.

Lichen was clean of impurities and smoothed. The lichen weighed (1,000 grams) was extracted by the maceration process. Lichen was immersed in methanol solvent for 3×24 hours, and then a filtering process was carried out to separate the filtrate and residue every 24 hours. The residue is macerated again with new methanol as solvent. The filtrate was combined and then concentrated using a rotary evaporator vacuum to obtain a methanol crude extract lichen Usnea sp.

2.3 Isolation and purification of lichen Usnea sp.

The initial separation stage uses the liquid-liquid extraction (LLE) technique. Methanol crude extract of lichen Usnea sp. is partitioned using a separating funnel with an increased polarity from nonpolar solvents to highly polar solvents (n-hexane, ethyl acetate, butanol, and water). This stage aims to separate compounds based on their level of polarity. The partition results were tested using a thin layer chromatography (TLC) method for searching and determining eluents with a specific ratio that could separate compounds well.

Furthermore, the subsequent separation was using gravity column chromatography (GCC) on the ethyl acetate fraction. The ethyl acetate fraction is put into the column; the separation process uses a mixed solvent whose polarity is different from polar to nonpolar solvent. The solvent used starts with 1 L n-hexane 100%, then a mixture of n-hexane:ethyl acetate and ethyl acetate:methanol in a gradient (v:v). All eluate results from GCC were tested on the TLC plate using nonpolar solvent and polar solvent in a gradient until there was specific color and calculating the Rf value under ultraviolet irradiation at 365 and 254 nm. The isolates were re-crystallized using n-hexane, ethyl acetate, and acetone as solvents. The isolated compound was analyzed using Fourier-transform infrared spectroscopy (FTIR) (Nicolet FT-IR iS10, USA) was used to observe a typical absorption band at the wavenumber and confirmed using nuclear magnetic resonance (NMR \textsuperscript{1}H and \textsuperscript{13}C) (JNM ECA 500 JEOL). We interpreted the data by comparing literature reviews and referring to the data obtained.

2.4 Glucosidase inhibitory activity

The antidiabetic test was carried out by inhibiting the α-glucosidase enzyme, according to Dewi et al. (2012)\textsuperscript{34}. Bioactivity testing was carried out using methanol crude extract, ethyl acetate fraction, n-hexane fraction, acetone fraction, and pure isolate compounds. Each test sample was made into four concentration series. The test sample was reacted with p-nitrophenyl-α-D-glucopyranoside and phosphate buffer solution pH 7.0, then incubated at 37°C for 5 minutes in a water bath. Each test sample was added with α-glucosidase enzyme solution and incubated at 37°C for 15 minutes in a water bath. The reaction was stopped by adding 1,000 μL of 0.2 M sodium carbonate solution. The amount of p-nitrophenyl released was then measured using UV-Vis spectrophotometry at 400 nm. Inhibitory activity is calculated by the equation as follows:

\[
\text{Inhibitory activity (\%)} = \frac{K - S}{K} \times 100
\]

Explanation:

K=Absorbance of negative control solution

S=Absorbance of test solution or positive control solution, where \(S = S_1\) (absorbance with the addition of enzymes) - \(S_0\) (absorbance without adding enzymes)

3 Results and Discussion

3.1 Isolation of lichen Usnea sp.

The phytochemical screening of the lichen extract was used to establish the classification of chemical compounds in lichen Usnea sp. Maceration against 1,000 grams of lichen Usnea sp. crude powder obtained 53 grams of methanol extract. Secondary metabolites identified positively in lichen Usnea sp. are alkaloid, flavonoid, and tannin. Ethyl acetate fraction was isolated by GCC to produce 90 sub-fractions. The stationary phase used silica
gel, and the mobile phase was a mixed eluent (n-hexane: ethyl acetate and ethyl acetate:methanol) (v:v). Further purification using TLC showed specific isolates. TLC was carried out using a solvent mixture of n-hexane:ethyl acetate (6:4) (v:v). TLC plate observations were carried out using UV lamps of 254 nm and 366 nm (Fig. 1). The single stain that appears on the TLC plate shows that isolate contains only one compound.

3.2 Identification of isolate structure

Pure isolate compounds isolated from the ethyl acetate fraction were identified for their functional groups using FTIR. The spectrum shows a typical absorption band in several regions of the wavenumber (Fig. 2). The absorption at wavenumber 3,091 cm⁻¹ is identified as a bond (-C-H sp²) supported by the absorption at wavenumber 1,630 cm⁻¹ which is characteristic (C=C) aromatic. The wavenumber 2,933 cm⁻¹ is identified as a bond (-C-H sp³). Typical absorption at 1,687 cm⁻¹ indicates the presence of a carbonyl group (C=O) aromatic ketone stretch. This absorption is slightly smaller than the typical carbonyl wave number because the carbonyl group (C=O) forms hydrogen bonds with the hydroxy group (OH), and there are bonds conjugation of a carbonyl group (C=O) with a bond (-C=C-) in the aromatic ring. The wavenumber 1,130 cm⁻¹ is thought to signal the ether group (C-O-C) bond stretching. The absorption of the hydroxy group (OH) did not appear suspected because the formation of hydrogen bonds between the carbonyl group (C=O) and the hydroxy group (OH) formed cyclic, which was considered more stable.

The determination number of carbon, hydrogen atoms and their positions was carried out using 1D NMR (¹H-NMR, ¹³C-NMR, and DEPT) and 2D NMR (HMQC and HMBC). The spectrum shows the similarity of data from usnic acid compounds. Based on a ¹H-NMR spectrum (Fig. 3), the isolate has two methyl groups with the multiplication of each singlet (-CH₃) at δH = 1.76 and 2.11 ppm. The shifts of 2.66 and 2.68 ppm indicate two methyl groups attached to the carboxylic group. The spectrum at the chemical shift δH = 5.98 ppm was identified as olefinic protons. The two hydroxy groups forming a hydrogen bond with the carbonyl group are shown by the chemical shifts δH = 13.32 and 11.04 ppm, respectively. At the H-16 position, the value of the methyl proton shift at the H-16 position is greater (downfield) due to the group position is directly attached to the phenolic group (Ar-OH) and the position that is also close to the hydroxy group (-OH) in position (C-8).

The ¹³C-NMR spectrum (Fig. 4) shows that the isolate has
18 carbon atoms, including three carbons from the carbonyl group (C=O) indicated by the shift value $\delta_C = 198.32$, 200.53, and 201.98 ppm. The presence of the carbonyl group (C=O) is strengthened by the presence of FTIR absorption bands at wavenumber 1,687 cm$^{-1}$. The four groups (-CH$_3$) are represented by chemical shifts at $\delta_C = 7.73$ ppm (C16), 28.11 ppm (C15), 31.49 ppm (C18), and 32.3 ppm (C13). The chemical shift value represents the group (-CH$_2$) at $\delta_C = 98.52$ ppm (C4). Thirteen quaternary carbon atoms consisting of 3 carbonyl groups, 3 C atoms that bind a hydroxy group, 2 C atoms that bind a methyl group, and five other quaternary C atoms are shown in sequence by peaks with a chemical shift value of 198.2 (C-1), 200.5 (C-14), 201.9 (C-17), 191.9 (C-3), 157.7 (C-10), 164.1 (C-8), 59.2 (C-12), 109.5 (C-9), 105.4 (C-2), 179.5 (C-5), 155.4 (C-6), 101.7 (C-11), and 104.1 ppm (C-7).

Based on HMQC data (Table 1), it is known that the aromatic protons at $\delta_H = 5.98$ ppm are bound to (C-4), while the proton peaks (CH$_3$) are at the chemical shift $\delta_H = 2.66$ (H-15), 2.68 (H-18), 2.11 (H-16), and 1.76 ppm (H-13) each bonded to the carbon atom at 28.1 (C-15), 31.5 (C-18), 7.7 (C-16), and 32.3 ppm (C-13).

HMBC (Table 1) shows data regarding the correlation between protons and their neighboring carbon atoms, which are 2 to 3 bonds apart. The resonance of aromatic protons at $\delta_H = 5.98$ ppm (H-4) is correlated with $\delta_C = 59.2$ (C-12), 105.4 (C-2), 179.5 (C-5), and 191.9 ppm (C-3). The methyl proton with a chemical shift value at $\delta_H = 1.76$ ppm (H-13) also correlates with the carbon atom at $\delta_C = 59.2$ (C-12), 179.5 (C-5), and with the carbonyl atom at the position $\delta_C = 198.2$ ppm (C-1). The methyl proton at $\delta_H = 2.66$ ppm (H-15) correlates with the carbon atom at $\delta_C = 105.4$ (C-2) and the carbonyl atom at $\delta_C = 200.5$ (C-14). Methyl proton at $\delta_H = 2.68$ (H-18) correlates with the carbon atom at $\delta_C = 104.1$ (C-7) and the carbonyl atom at $\delta_C = 201.9$ (C-17). The methyl proton at $\delta_H = 2.11$ (H-16). The proton of the hydroxy group (-OH) attached to the carbon atom at position $\delta_C = 164.1$ (C-8) correlates with $\delta_C = 104.1$ (C-7), 109.5...
In the final stage, we compare results with the appropriate literature by Huneck and Yoshimura (1996) and Maulidiyah et al. (2021), it is assumed that the isolated compound from the ethyl acetate fraction is a usnic acid (C18H16O7) (Table 2). Usnic acid is one of the main contents of lichen Usnea sp. which has many benefits in the pharmacological field. The molecular structure of the usnic acid compound is shown in Fig. 5.

3.3 Effect of lichen metabolites on α-glucosidase inhibition

The inhibition of α-glucosidase by quercetin, lichen Usnea sp. extract, and usnic acid compounds have been evaluated (Table 3). Quercetin acts as a positive control having a high inhibitory power compared to lichen Usnea sp. extract and usnic acid with IC50 values of 5.69, 88.02, and 106.78 μg/mL, respectively. The usnic acid compound has a moderate performance in its activity to inhibit the α-glucosidase enzyme.
Lichen *Usnea* sp. extract shows excellent stability because it still contains various chemical compounds that work simultaneously to inhibit the $\alpha$-glucosidase enzyme, whereas pure usnic acid compound is a single compound and doesn’t contain many hydroxy groups, which play an active role in inhibiting the $\alpha$-glucosidase enzyme. Although it shows moderate performance in inhibiting the $\alpha$-glucosidase enzyme, it has a potential positive impact as an antidiabetic. In addition, the antidiabetic activity of usnic acid compounds from lichens *Usnea* sp. is not much reported about the inhibition of the $\alpha$-glucosidase enzyme (Table 4).

### Table 3 Data test results for $\alpha$-glucosidase enzyme inhibitory activity.

| Sample                  | Concentration (µg/mL) | Inhibition (%) | IC$_{50}$ (µg/mL) |
|-------------------------|-----------------------|----------------|-------------------|
| Positive control of quercetin | 10                    | 78.81          | 5.69              |
|                         | 7.5                   | 60.80          |                   |
|                         | 5                     | 14.55          |                   |
|                         | 2.5                   | 28.59          |                   |
|                         | 100                   | 46.04          |                   |
| Lichen *Usnea* sp. extract | 50                    | 22.43          | 88.02             |
|                         | 25                    | 4.92           |                   |
|                         | 10                    | 1.68           |                   |
|                         | 25                    | 18.40          |                   |
|                         | 10                    | 1.71           | 106.78            |
| Usnic acid              | 5                     | $-1.79$        |                   |

### Table 4 Reported lichen and its compounds as antidiabetic agents $\alpha$-glucosidase.

| Lichens            | Compounds                        | Activity            | Authors [Ref]           |
|--------------------|----------------------------------|---------------------|-------------------------|
| Ramalina celastri  | Usnic acid                       | $\alpha$-Glucosidase| Verma *et al.*, 2012$^{25}$ |
| Ramalina nervulosa | Sekikaic acid                    | $\alpha$-Glucosidase| Verma *et al.*, 2012$^{25}$ |
| Ramalina pacifica  | Salazinic acid                   | $\alpha$-Glucosidase| Valadbeigi, 2016$^{26}$  |
| R. nervulosa       | Sekikaic acid                    | $\alpha$-Glucosidase| Verma *et al.*, 2012$^{25}$ |
| Caloplaca biatorina| -                                | $\alpha$-Glucosidase| Thadhani *et al.*, 2011$^{27}$ |
| Cladonia sp.       | Zeorin                           | $\alpha$-Glucosidase| Karunaratne *et al.*, 2014$^{29}$ |
|                    | Methyl-$\beta$-orcinol carboxylate| $\alpha$-Glucosidase| This Work               |
| T. flavicans       | Vicanicin                        | $\alpha$-Glucosidase| Maulidiyah *et al.*, 2020$^{29}$ |
| Usnea sp.          | Usnic acid                       | $\alpha$-Glucosidase| This Work               |
This research provides a perspective on the source of natural compounds from lichen *Usnea* sp. as antidiabetic drugs in the future.

**Author Contributions**

M.M. and W.W. performed all the experiments. A.D. coordinated the study. M.N writing the manuscript. A.M. and L.O.A.S processed the research data. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest**

The authors report no financial or any other conflicts of interest in this work.

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