Antioxidant activity-guided isolation of usnic acid and diffractaic acid compounds from lichen genus *Usnea* sp.

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**ABSTRACT**

The unique secondary metabolites from the lichen organism have been utilized as a raw material for producing natural medicine, which is very useful as an antioxidant (AO) agent. As we know, it also varies greatly depending on the surrounding environment. In this study, we explore the secondary metabolites from the lichen organism genus *Usnea* sp. originating from Southeast Sulawesi, Indonesia (tropical country), through an isolation procedure and its AO activity test. Based on the research results, the secondary metabolites from lichen *Usnea* sp. have been successfully isolated based on 1D-NMR spectroscopic interpretation, showing that the isolated compounds contain usnic acid (C₇,H₁₈,O₇, yellow crystal) and diffractaic acid (C₁₇,H₁₈,O₇, white crystal) in acetone extract. Moreover, the AO activity test using the 1,1-diphenyl-2-picryl-hydrazine method exhibits that diffractaic acid has quite an activity as AO agent, while usnic acid and acetone extract have provided the highest superoxide anion radical-scavenging activity as an AO agent. These chemical compounds are potentially used as a natural medicinal ingredient as an AO agent.

**INTRODUCTION**

In recent years, the development of natural medicine has shown interesting attention to be applied to various cell generation treatments (Langer and Tirrell, 2004). It is due to the fact that there are no harmful effects on the human body and it can slowly improve cell generation. Today, the community is very concerned about maintaining a healthy and hygienic body in order to avoid viruses/bacteria and eradicate free radicals (Pompilio et al., 2013). Moreover, the coronavirus disease 2019 pandemic has increased the vigilance of people around the world to always maintain their health (Greenberg et al., 2020). Thus, it is important to maintain a healthy body in order to avoid various diseases by utilizing natural compounds from nature. Several secondary metabolites of lichen have attracted the attention of researchers to be used as supplements to maintain the health of the human body. In addition, it is widely used in antimicrobial ointments as an antioxidant (AO) agent to destroy microbial cell membranes (Gülçin et al., 2002). The AO will also inhibit or delay the oxidation process through the initiation or propagation by oxidation reaction (free radicals) (Riley, 1994; Taghvaei and Jafari, 2015). The unique AO compounds in lichen have a very good impact on society.

Hamza Sherif and Gebreyohannes (2018) and Ajileye et al. (2015) have reported that the discovery of AO compounds can be obtained through two pathways, namely the synthesis of chemical compounds and isolation from natural ingredients. These pathways also have advantages and disadvantages to obtain AO ingredients; sometimes, the target compound is difficult to be isolated by considering factors including environmental, sterilization of tools and materials, and solvents (Zhai et al., 2013). In addition,
the discovery of AO through the isolation process from natural products, such as plants and other organisms, is a more attractive choice because the synthetic AO is reported to be carcinogenic.

Several AO compounds have been extracted by researchers from natural products, such as rosehip species (Fascella et al., 2019), Buriti (Mauritia flexuosa L.f.) (Resende et al., 2019), Agaricus bisporus (Ramos et al., 2019), bamboo (Nirmala et al., 2018), tropical fruits (Albuquerque et al., 2019), and Sida rhombifolia leaves (Ferro et al., 2019). Apart from the above-mentioned sources, lichen is reported as another source of AO with high bioactivity (Ahamed et al., 2019). Lichen is an organism formed through the mutualism symbiotic between fungi and algae (cyanobacteria) (Honegger et al., 2013). It can be found in the pine forest and is very sensitive to environmental disturbances and can be used to cheaply assess air pollution (Gilbert, 1973; Neurohr Bustamante et al., 2013).

Particularly, Indonesia has a high biodiversity and has been classified as a tropical country. Based on this, various kinds of biodiversity are resistant to environmental conditions (Arifin and Nakagoshi, 2011). It also applies to lichen organisms which are strongly influenced by the environment because they are widely used as environmental indicators or bioindicators. Several studies have reported that the lichen organisms absorb chemical substances dissolved in water. If the air is very badly polluted with sulfur dioxide, there may be no lichens present (Nash, 1976). On the other hand, lichens have also been observed by researchers as AO agents such as Ramalina conduplicans (Luo et al., 2010), Ramalina terebrata (Paudel et al., 2011; Kim et al., 2018), Cladonia (Aslan et al., 2006; de Barros Alves et al., 2014; Kosanić et al., 2014a), Evernia prunastri and Pseudoevernia furfuracea (Kosanić et al., 2013), Parmelia (Gulluce et al., 2006; Manojlović et al., 2012), and Cladonia planus (Hassan et al., 2013). The AO compounds from lichen were discovered like fumarprotocetraric (de Barros Alves et al., 2014), protocetraric acid (Tay et al., 2004), depside, dibenzofuran, dibenzoquinone (Huovinen and Ahti, 1982), Ramalin (γ-glutamyl-N'- (2-hydroxyphenyl)hydrazide) (Kim et al., 2018; Paudel et al., 2011), salazinic acid (Ingólfsdóttir et al., 1998), and atranorin (Dahlquist and Fregert, 1980; Bačkorová et al., 1998). Although they show good bioactivity as AO agents, they are also needed on various compounds test to determine their benefits for humans.

Based on our previous study, the lichen Usnea sp. contains secondary metabolite compounds such as 3-[1’-(2”,3”-dihydroxyphenyl)-propyl]-7-hydroxy-chroman-4-one (Maulidiyah et al., 2018), (SE,6E) 5-ethylidene-7-formyl-6,7-hydroxy methyl hept-6-enoate (Maulidiyah et al., 2016b), Eumitrin A1 (Maulidiyah et al., 2015), 2’-hydroxy-1’-(4-hydroxy-5-methoxy-2-methyl-phenyl)-etone (Maulidiyah et al., 2011), and atranorin (Maulidiyah et al., 2016a). In this study, we explore the potential of AO activity against usnic acid and diffractaic acid compounds based on the lichen genus Usnea sp., originating from Southeast Sulawesi, Indonesia. The isolation of organic compounds (usnic acid and diffractaic acid) and their AO test will be discussed in detail.

**EXPERIMENTAL METHOD**

**Extraction and purification of lichen Usnea sp.**

Isolation of secondary metabolites from lichen Usnea sp. was conducted by considering the steps including the extraction, separation, and purification. Firstly, in the sample preparation, the lichen was collected from the pine forest in Moweve district, Southeast Sulawesi, Indonesia. It was cleaned and dried at room temperature and then crushed into small pieces to optimize the maceration process. After that, the lichen was macerated for $3 \times 24$ hours using an acetone solvent (Merck, Germany) and filtered every 24 hours to obtain an acetone extract from the lichen. The separation step was conducted using column chromatography gravity (Pyrex) in the stationary phase with silica gel 60G (0.063–0.200 mm) and eluted gradient with n-hexane and ethyl acetate solvents (Merck, Germany), whereas in the purification process, it was fractionated by using Thin-layer chromatography (TLC) under the acetone extract to observe the spot stains which indicates that the compound was pure. Based on nine fractions (Fig. 1), we observe that the fractions of 5 (L5) and 7 (L7) exhibit the formation of a crystal needle and are characterized using a 1D-NMR spectrometer (JEOL JNM ECA 500).

**Antioxidant activity test**

The AO activity test was applied using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method based on those reported by Kosanić et al. (2014b), where 2 ml methanol and 0.05 mg/ml DPPH solutions were added into a glass cuvette and mixed with sample concentrations of 1, 5, 10, and 15 μg.ml$^{-1}$, respectively. The solution was mixed and shaken vigorously at an ambient temperature, and then incubated for 30 minutes. Finally, it was analyzed using UV-vis spectrophotometer (UV-2550, Shimadzu, Japan) on $\lambda_{max} = 515$ nm and its AO capacity was calculated by referring to the following equation (Kosanić et al., 2014b):

$$\% \text{Antioxidant} = (A_b - A_n) A_n \times 100 \quad (1)$$

where $A_b$ is the absorbance of the negative control and $A_n$ is the absorbance of the reaction mixture or standard. Quercetin was used as the positive control in this treatment.

**RESULTS AND DISCUSSION**

**Identification of secondary metabolite compounds**

Isolation of secondary metabolites from lichen Usnea sp. was successfully isolated by the isolation procedure using the TLC technique to observe a single color spot on the TLC plate showing the acquisition of pure compound (Fig. 1). It can be seen that there were nine fractions, but only two fractions show a single color spot, namely L5 and L7, with yellow and white crystals (Fig. 2). To confirm this, the secondary metabolite compound on L5 was evaluated using 1D-NMR spectroscopy (1H proton and 13C carbon) as shown in Figure 3.

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**Figure 1.** TLC of nine lichen Usnea fractions.
**Figure 3A** shows that the L5 fraction contains 18 carbon atoms in a chemical shift at 201.21, 200.97, and 191.28 ppm which describes three carbon atoms originating from the carbonyl group (C = O) and chemical shift at 7.57, 19.51, 27.89, 31.11, and 31.51 ppm clarified from the methyl group (CH₃). In addition, the methin group was identified based on one carbon atom with a chemical shift at 98.36 ppm and nine quaternary carbons with chemical shifts at 62.54, 156.77, 155.35, 101.19, 98.36, 107.17, 105.42, 104.93, and 58.61 ppm. Based on these results, there have been five carbon atoms bonded to oxygen atoms.

Moreover, the 1H-NMR (Fig. 3B) was also analyzed toward the L5 fraction, which indicates the existence of four methyl groups with chemical shifts at 0.84, 1.22, 1.73, and 1.99 ppm. There are two other methyl groups that bind the C=O group that has been identified in chemical shifts at 2.58 and 2.66 ppm. The chemical shift at 6.30 ppm indicates the presence of the olefinic group. In the final stage, we compare these data with the results of previous studies by Maulidiyah *et al.* (2011) and Huneck and Yoshimura (1996), showing that L5 has similarities with the usnic acid compound (chemical formula C₁₈H₁₆O₇) (Table 1).

![Image of crystals](image-url)

**Figure 2.** The formation of crystals (L5 = yellow; L7 = white).

**Table 1.** Comparison of L5 isolated compound with usnic acid compounds based on the value of chemical shift of the proton and carbon.

| No | ¹³C-NMR (ppm) | ¹³C-NMR (ppm) | ¹³C-NMR (ppm) | ¹³C-NMR (ppm) |
|----|---------------|---------------|---------------|---------------|
| 1  | 198.06        | –             | –             | –             |
| 2  | 105.24        | –             | 105.42        | –             |
| 3  | 191.70        | –             | 191.28        | –             |
| 3-OH | –          | 18.85 (1H, s) | –             | –             |
| 4  | 98.31         | 5.98 (1H, s)  | 98.37         | 6.30 (s)      |
| 4a | 179.37        | –             | 179.37 seconds | –             |
| 5a | 155.21        | –             | 155.36        | –             |
| 6  | 101.53        | –             | 101.20        | –             |
| 7  | 163.91        | –             | 162.54        | –             |
| 7-OH | –             | 11.00 (1H, s) | –             | 11.33 (br-s)  |
| 8  | 109.35        | –             | 107.18        | –             |
| 9  | 157.51        | –             | 156.78        | –             |
| 9-OH | –             | 13.30 (1H, s) | –             | –             |
| 9a | 103.94        | –             | 104.94        | –             |
| 9b | 59.08         | –             | 58.62         | –             |
| 10 | 201.73        | –             | 201.22        | –             |
| 11 | 32.09         | 1.76 (3H, s)  | 31.51         | 1.73 (s)      |
| 12 | 27.51         | 2.67 (3H, d, J = 2.4 Hz) | 27.90 | 2.67 (d) |
| 13 | 7.51          | 2.51 (3H, s)  | 7.58          | 2.50 (s)      |
| 14 | 200.29        | –             | 200.98        | –             |
| 15 | 31.22         | 2.67 (3H, d, J = 2.4 Hz) | 31.11 | 2.67 (d) |

-ur- (Unrecorded).

*Adapted from Ivanova *et al.* (2004).
In the same stage, we also analyze the fraction of L7 by using 1D-NMR to identify a secondary metabolite compound in the white crystal needle. Figure 4 shows $^{13}$C-NMR and $^1$H-NMR data from the L7 fraction that presents 20 carbon atoms, wherein two carbon atoms have been bound to the carbonyl group with chemical shifts at 175.41 and 167.98 ppm. Nine quaternary carbon atoms were identified at 164.40, 161.67, 158.47, 154.38, 141.58, 136.75, 121.11, 118.51, and 117.77 ppm and five of them bind directly to oxygen atoms. Moreover, two carbon atoms were expressed as methyl groups and two other carbons as methoxy groups with chemical shifts at 116.99, 109.36, 62.71, and 56.41 ppm. The presence of four carbons methyl groups was strengthened by chemical shifts at 24.13, 20.26, 9.47, and 9.19 ppm, respectively.

It was clarified based on hydrogen atoms ($^1$H) with chemical shifts at 3.79 (3H, s); 3.84 (3H, s); 2.09 (3H, s); 2.11 (3H, s); 2.41 (3H, s); and 2.55 (3H, s) ppm. The other hydrogen atoms also appeared at 6.52 (1H, s) and 6.66 (1H, s) ppm, indicating the presence of two protons; each binding to a different aromatic ring. As presented in Table 2, we conclude that the L7 fraction has similarities with the diffractaic acid (C$_{20}$H$_{22}$O$_7$) compound, as previously reported by Kumar and Müller (1999) and Huneck and Yoshimura (1996).

**Antioxidant test**

The AO activity test over acetone extract, usnic acid, and diffractaic acid compounds was conducted by the scavenging DPPH radical method. The role of AO testing was to observe the
influencing isolated compounds with acetone extract. Hodzic and Pasalic (2009) exhibited that the AO activity test was effectively observed based on 50% inhibition concentration (IC$_{50}$) (Table 3). In this test, we use quercetin as a positive control to compare in AO testing of test compounds. In addition, we present the linearity equation from variation concentrations of samples (Fig. 5). The isolated lichen compounds demonstrated a higher IC$_{50}$ value compared with quercetin as a positive control with an IC$_{50}$ value of 5.12 μg/ml. The lower the IC$_{50}$ value of a sample, the greater the ability as an AO. Based on Table 3, it can be concluded that the high activity test is consecutive, namely quercetin, usnic acid, acetone extract, and diffractaic acid.

The scavenging of superoxide anion radicals by the tested acetone extract and compounds shows a statistically significant difference between samples and control ($p < 0.05$) (Kosanić et al., 2014b). The usnic acid showed the highest superoxide anion radical-scavenging activity compared with the acetone extract and diffractaic acid (the IC$_{50}$ was 7.67 μg.ml$^{-1}$). It is due to the fact that

Figure 4. NMR spectra of the L7 fraction compound: (A) $^{13}$C-NMR spectrum and (B) $^1$H-NMR spectrum.
Table 2. Comparison of L7 isolated compound with diffractaic acid compounds based on the value of chemical shift of the proton and carbon.

| No | Diffractaic acid | L7 Compound (I) |
|----|-----------------|-----------------|
|    | \(^{13}\text{C-NMR}\) (ppm) | \(^{13}\text{C-NMR}\) (ppm) |
|    | \(^{1}H\text{-NMR}\) (\(\sum H\), mult., \(J\) in Hz) | \(^{1}H\text{-NMR}\) (\(\sum H\), mult., \(J\) in Hz) |
| 1  | 119.73          | 118.51          |
| 2  | 160.32          | 161.68          |
| 3  | 117.68          | 117.77          |
| 4  | 157.44          | 158.47          |
| 5  | 108.38, 6.63 (1H, s) | 109.36, 6.66 (s) |
| 6  | 135.76          | 136.75          |
| 7  | 166.56          | 168.00          |
| 8  | 9.12, 2.17 (3H, s) | 9.19, 2.11 (s)  |
| 9  | 20.45, 2.48 (3H, s) | 20.26, 2.41 (s) |
| 1' | 117.81          | 116.99          |
| 2' | 164.07          | 164.40          |
| 3' | 108.71          | 111.73          |
| 4' | 154.41          | 154.38          |
| 5' | 116.99, 6.63 (1H, s) | 116.99, 6.52 (s) |
| 6' | 141.39          | 141.59          |
| 7' | 176.20          | 175.42          |
| 8' | 9.36, 2.19 (3H, s) | 9.48, 2.09 (s)  |
| 9' | 24.39, 2.61 (3H, s) | 24.13, 2.58 (s) |
| 2-\text{OCH}_3| 55.94, 3.86 (3H, s) | 56.42, 3.79 (s) |
| 4-\text{OCH}_3| 62.35, 3.87 (3H, s) | 62.72, 3.85 (s) |

\(^a\)Adapted from Bayir et al. (2006).

Table 3. IC\(_{50}\) value of the isolate compound of L5 (usnic acid) and L7 (diffractaic acid).

| Test samples       | IC\(_{50}\) (\(\mu\)g/ml) |
|--------------------|--------------------------|
| Quercetin          | 5.12                     |
| Acetone extract    | 9.42                     |
| Usnic acid         | 7.67                     |
| Diffractaic acid   | 18.51                    |

\(^a\)All data were statistically significant with \(p < 0.05\).

USNIC acid is contained over the phenolic groups (-OH) which are very reactive compounds. In fact, it was observed that the examined USNIC acid containing a higher content of phenols exerted a stronger radical-scavenging effect, suggesting that phenolics are the main agents responsible for their AO activity (Kosanić et al., 2014b). Previously, the AO properties of USNIC acid were also reported by Manojlović et al. (2012), wherein the USNIC acid showed strong AO properties with IC\(_{50}\) values \(p < 50 \mu\)g.ml\(^{-1}\).
CONCLUSION

Isolation and AO activity tests were successfully examined to obtain the chemical composition of acetone extract of the lichen genus *Usnea* sp. originally from Southeast Sulawesi, Indonesia. Based on the isolation and purification methods, the two secondary metabolites, namely usnic acid and diffractaic acid, by physical properties (color) as yellow and white crystal needles were discovered. In addition, the usnic acid compound has a strong AO compared to acetone extract and diffractaic acid. It is due to the fact that the strong AO activity of usnic acid is correlated with a high content of total phenols. These chemical compounds are potentially used as a natural medicinal ingredient as an AO agent.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

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

ETHICAL APPROVALS

This study does not involve the experimentation on animal or human subjects.

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