RJC

**RASĀYAN *J. Chem.***
Vol. 15 | No. 4 |2685-2691| October - December | 2022
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

# SECONDARY METABOLITE ISOLATION FROM INDONESIAN FOLIOUS LICHEN: *Phyloporon aciculare* (Ach.) Nyl

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**ABSTRACT**

Lichen has been investigated for discovering new antibacterial agents. Although Indonesia is considered one of the megadiverse countries and a home to a diverse array of lichen species, research on lichens for medicinal properties remains scarce. In this study, an intensive semi-preparative High-Performance Liquid Chromatography successfully isolated a major compound from *Phyloporon aciculare*, with its molecular structure proposed as methyl 2,4-dihydroxy-3,6-dimethylbenzoate by Nuclear Magnetic Resonance spectral and was confirmed by Mass Spectrometry data analysis. The crude extract of the lichen possessed a selective antibacterial activity in which significantly inhibited *Kocuria rhizophila* by 96.9% at a 100 µg/mL concentration. In addition, previous reports on methyl 2,4-dihydroxy-3,6-dimethylbenzoate from other species indicated the compound to have antibacterial, anti-inflammatory, and anticancer activities.

**Keywords:** *Phyloporon aciculare*, Depside, Antibacterial, Atraric Acid, Lichen.

RASĀYAN *J. Chem.*, Vol. 15, No.4, 2022

## INTRODUCTION

Infectious diseases caused by pathogenic bacteria, parasites, viruses, and fungi have been serious health threats throughout human history.[1, 2] Meanwhile, most people in low-income countries seem to be prone to the severe impact of infectious diseases.[3] Global society is facing antimicrobial resistance that demands the discovery of new antibacterial agents.[4-7] Natural product has been an enormous source of medicine, including antibacterial agents.[8] The discovery of penicillin from Penicillin fungi has led to the search for new antibacterial from fungi-related organisms, including fungi-algae composite organisms, lichen since the 1940s.[9, 10] Nevertheless, Burkholder *et al.* discovered that 27 of 42 different lichen extracts had antibacterial activity against pathogenic *Bacillus subtilis* and *Staphylococcus aureus* in preliminary


http://doi.org/10.31788/RJC.2022.1548027

studies.[11] Lichen is a cosmopolitan organism with a high capacity to adapt to diverse environments, from polar to tropical climates.[12,13] Although more than 20,000 lichen species have been identified worldwide from various habitats; enormous numbers remain unidentified and understudied.[14,15] Similarly, in Indonesia, some lichens have been used for traditional medicine, but a large number of lichens from this archipelagic country are not well studied.[16,17] In this presented study, phytochemical and pharmacological procedures were performed to isolate and evaluate the antibacterial activities of understudied *Phyloporon aciculare* (Fig.-1), from which the results were reported for the first time.

Fig.-1: *Phyloporon acicular* (Ach.) Nyl Attached to the Stone

## EXPERIMENTAL

### Lichen Collection

The lichen *Phyloporon aciculare* grown on rock surface was collected by T.A.L. in Mount Lawu Surroundings at Sarangan, Magetan Regency, East Java, Indonesia with a geographical coordinate of 7°39'51.1S111°11'34.0E. The lichen biomass was transported to Drug Utilisation and Discovery Research Group (DUDRG), Faculty of Pharmacy-University of Jember, Indonesia with a voucher code G1. A voucher copy sample was sent to a lichenologist, L.F.U., at Gadjah Mada University's Faculty of Biology in Yogyakarta, Indonesia for identification.

### Extraction and Isolation

Dried lichen samples were ground into a powder. The sample powder of 50 g was mixed with 150 mL methanol and was then stirred at 200 rpm for 24 hours at room temperature. The filtrate was separated and vacuum dried to produce crude methanol extract. The process was repeated six times to gain 0.86 g dried crude methanol extract. A portion of 0.2 g crude extract was mixed in 4 mL methanol and was filtered through a Polytetrafluoroethylene membrane filter (PTFE, 0.45 µm) before loading into Cecil HPLC system in 27 block injections of 100 µL. The semi-preparative High-Performance Liquid Chromatography (HPLC) system was composed of a CE 4104 pump, a CE 4040 degasser, and a CECIL CE4300 detector controlled by a CE4900 with PowerStream software. A reverse phase column (10 x 250mm 4 µm 80, YMC Sphere ODS M80) was employed. The elution gradient started from 0-20% solvent B (acetonitrile) within 25 minutes, followed by 20-50% solvent B (solvent A: milli-Q water, solvent B: acetonitrile) within 5 minutes at a flow rate of 2 mL/min. A chromatogram was produced at $\lambda$ 254 nm. A major constituent was isolated around 4.0 mg with a retention time of 13 minutes 51 seconds and was identified as methyl 2,4-dihydroxy-3,6-dimethylbenzoate or atraric acid.

### Molecular Structure Characterization

The sample of 4.0 mg dried pure isolate was dissolved in deuterated acetone ($(CD_3)_2CO$) and was loaded into the nuclear magnetic resonance (NMR) module, where measurements were collected at 500 MHz for $^1$H-NMR and 125 MHz for $^{13}$C-NMR resonant frequencies. Mnova software version 14.1 was used to process the 1D-NMR and 2D-NMR spectral data. Electrospray Ionization Mass Spectrometry (ESI-MS) data from a Shimadzu LC-2010 Mass Spectrometer confirmed the molecular formula.

### Antibacterial Assay

Antibacterial activity of *n*-hexane lichen fraction was performed using a microdilution method with a single concentration of 100 µg/mL per the Clinical and Laboratory Standard Institute's standard protocol.[18] The absorbance of the bacterial suspension was measured through a spectrometer at $\lambda$ 625 nm and was adjusted

to the 0.5 Mc Farland standard, followed by dilution at 100 folds to yield 1 x $10^6$ CFU/mL. The antibacterial activity was determined by allocating 50 µL bacterial suspension into microplate wells with a crude extract (100 µg/mL in 1.0 % dimethyl sulfoxide (DMSO) in Cation-Adjusted Mueller Hinton Broth (CAMHB), 50 µL) or positive control (200 µg/mL gentamicin in CAMHB, 50 µL) or negative control (1.0 % DMSO in CAMHB, 50 µL). CAMHB was also loaded into the well as a media control until the final volume became 100 µL. The test control was a mixture of *n*-hexane fraction and media, as well as gentamicin and media. The test material was placed in a 96-well microplate and was then incubated at 37°C for 20 hours using an incubator shaker. The test results (100 µg/mL) were recorded using a microplate reader set to λ 625 nm (molecular devices BIORAD-Benchmark M550 microplate reader). *Pseudomonas auruginosa* ATCC 27853, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25992, *Bacilus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538 were used for the antibacterial activity test. The antibacterial activity was indicated as a percentage inhibition based on a calculation using the equation below (P: negative control, Q: media control, R: test sample, S: control). Inhibition percentage data significance was evaluated based on Student's *t*-test using the IBM SPSS Statistics 22 software (*p*-value <0.05 indicates a statistically significant result).

$$\text{Percentage Inhibition} = \left(1 - \frac{(Abs.R - Abs.S)}{(Abs.P - Abs.Q)}\right) x100\%$$

## RESULTS AND DISCUSSION

The understudied lichen *P. aciculare* (Ach.) Nyl was a composite organism collected from the rock of Mount Lawu at an elevation of approximately 1900 meters above sea level. The Lawu terrane region provides an ideal environment for this folious lichen to grow and produce a large amount of biomass for phytochemical studies. In this study, an extensive chromatographic procedure successfully yielded a pure major constituent based on the purity test indicated by the chromatogram below (Fig.-2). In addition, the brown crystalline isolate produced clear and clean NMR spectral properties (Fig.-3).

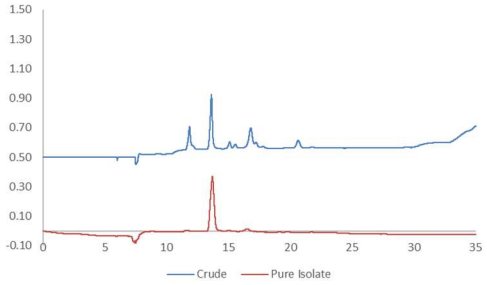


Fig.-2: Chromatogram of Crude Extract (top) and Purity Test Result of Major Isolate of *P. aciculare*

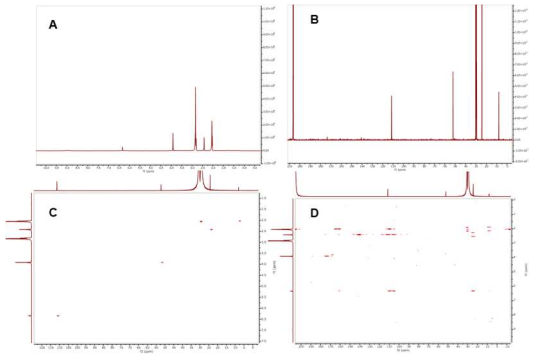


Fig.-3: $^1$H-NMR (A), $^{13}$C-NMR (B), gHSQC (C), and gHMBC (D) spectra of depside from *P. aciculare*

The $^1$H-NMR spectra analysis showed a typical signal of hydrogen bonding at $\delta$ 11.98 ppm, a distinct signal formed between the hydroxyl group (C2-OH) and the carbonyl group (C = O). The singlet signal at $\delta$ 6.36 ppm (s, 1H, H5) in the $^1$H-NMR spectrum with proton integration of one, suggested a single hydrogen attached to a benzene ring in which heteronuclear single quantum coherence (gHSQC) spectral analysis

indicated the proton to correlate with carbon signal at $\delta$ 111.54 ppm assigned to C5. Further Heteronuclear Multiple Bond Correlation (gHMBC) spectral analysis indicated a proton-carbon correlation between proton signal at $\delta$ 6.36 ppm (s, 1H, H5) and carbon resonated at $\delta$ 173.54 ppm (C7), $\delta$ 104.84 ppm (C1), and $\delta$ 24.19 ppm (C6-$CH_3$). Furthermore, $^1$H-NMR spectral analysis showed the presence of a methoxy group at a chemical shift at $\delta$ 3.88 ppm (s, 3H, C7-OC$\underline{H}_3$) in which gHMBC spectral analysis showed this proton to correlate with carbon signal at $\delta$ 104.84 ppm assigned to C1. Further gHMBC spectral analysis, proton resonated at $\delta$ 2.42 ppm (s, 3H, C6-$CH_3$) assigned to methyl group attached to carbon C6 with chemical shifts of $\delta$ 140.52 (C6). The gHMBC of signal $\delta$ 2.42 ppm (s, 3H, C6-$CH_3$) correlate with resonances at $\delta$ 104.84 (C1), $\delta$ 111.54 (C5), and $\delta$ 173.54 (C7). This spectral analysis suggested construction of fragment A of the molecule as shown in Fig.-4A. On the other hand, $^1$H-NMR spectral analysis showed a singlet signal at $\delta$ 2.05 ppm (s, 3H, C3-$CH_3$), with the integration of three protons in which its downfield chemical shift indicating this methyl attached to a benzene ring. This was supported by gHMBC spectral analysis which the proton correlates with carbon signal at $\delta$ 109.47 ppm assigned to C3. Further gHMBC analysis showed the methyl proton to have three bond lengths of proton-carbon correlation against carbon peak resonated at $\delta$ 164.08 ppm and $\delta$ 161.08 ppm, assigned to C2 and C4, respectively. The downfield carbon signal suggested the aromatic carbon has an adjacent hydroxyl group. This spectral analysis suggested construction of fragment B of the molecule as shown in Fig.-4B. Further gHMBC spectral analysis showed the signal $\delta$ 6.36 ppm (s, 1H, H5) also correlate with $\delta$ 109.47 ppm (C3), $\delta$ 8.06 ppm (C3-$\underline{C}H_3$), and $\delta$ 161.08 ppm (C4). In addition, gHMBC spectral analysis showed the singlet signal $\delta$ 2.05 ppm (s, 3H, C3-C$\underline{H}_3$) and $\delta$ 2.42 ppm (s, 3H, C6-C$\underline{H}_3$) to have resonance with carbon signal at $\delta$ 140.52 ppm (C6) and $\delta$ 164.08 ppm (C2), respectively. This NMR spectral analysis was able to support a connection between fragments A and B, therefore constructing a molecular structure of a major isolate of *P. aciculare* in which proton-carbon correlation was summarized in Fig.-4C below.

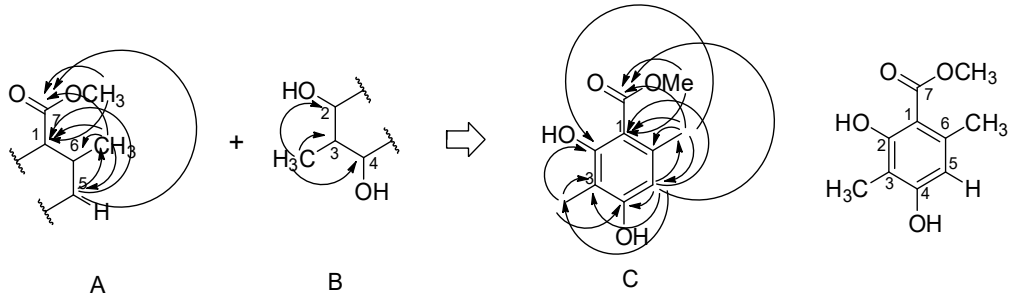


Fig.-4: Fragmented the Molecular Structure Analysis of the Major Compound of *P. aciculare* Compound Based on NMR Spectral Data

The molecular structure of the major compound of *P. aciculare* was further confirmed through chemical shift comparison against literature data of the same compound isolated from the lichen, *Parmotrema mesotropum* (Parmeliaceae family).[19] According to the literature study and the NMR data analysis (Table-1), the major compound isolated from *P. aciculare* was methyl 2,4-dihydroxy-3,6-dimethylbenzoate, a depside compound with the molecular formula of $C_{10}H_{12}O_4$. The molecular formula was confirmed by the LRMS $[M+H]^+$ signal at *m/z* 197, as shown in Fig.-5. Depsides are the most abundant lichen's secondary metabolites in which its monomers commonly form distinct lichen chemotypes such as depside, depsidone, and dibenzofuran with or without intramolecular cyclisation.[20]

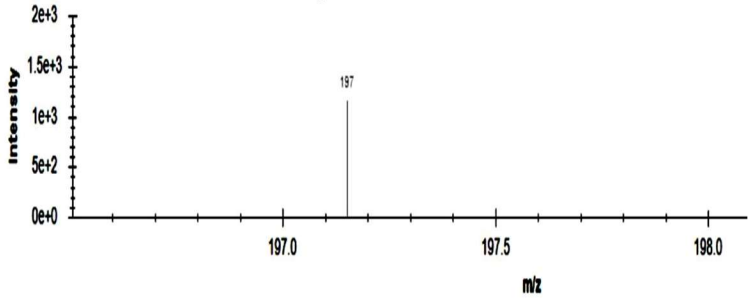


Fig.-5: LMRS Major Compound of Lichen *P. aciculare*

Table-1: Comparison $^{1}H$ and $^{13}C$ NMR Spectroscopic Experimental dan Literature

| No. | | Experimental | | Literature [19] | |
|---|---|---|---|---|---|
| | | $\delta_H$ (500 MHz, $(CD_3)_2CO$) | $\delta_C$ (125 MHz, $(CD_3)_2CO$) | $\delta_H$ (600 MHz, $CDCl_3$) | $\delta_C$ (150 MHz, $CDCl_3$) |
| 1 | C1 | - | 104.84 | - | 105.18 |
| 2 | C2 | - | 164.08 | - | 163.08 |
| 3 | C3 | - | 109.47 | - | 108.55 |
| 4 | C3-$CH_3$ | 2.05 (s, 3H) | 8.06 | 2.104 (s, 3H) | 9.35 |
| 5 | C4 | - | 161.08 | - | 158.05 |
| 6 | C5 | 6.36 (s, 1H) | 111.54 | 6.209 (s, 1H) | 110.55 |
| 7 | C6 | - | 140.52 | - | 140.14 |
| 8 | C6-$CH_3$ | 2.42 (s, 3H) | 24.19 | 2.460 (s,3H) | 24.06 |
| 9 | C7 | - | 173.54 | - | 172.61 |
| 10 | C7-$OCH_3$ | 3.88 (s, 3H) | 52.12 | 3.921 (s, 3H) | 51.81 |

Crude extract of hexane fraction was tested against several microbes. The crude *n*-hexane fraction possessed the highest inhibitory activity against *Kocuria rhizophila* with a percentage of inhibition value of 96.6 ± 2.9% with *p*-value < 0.05 at a concentration of 100 µg/mL. The crude extract indicated moderate antibacterial activity against *Pseudomonas aeruginosa* and low inhibition against *Bacillus subtilis, Escherichia coli, Bacillus cereus,* and *Staphylococcus aureus* (Table-2).

Table-2: Antibacterial Inhibition Percentage at 100 µg/mL

| Bacteria | Inhibition (%) |
|---|---|
| *Pseudomonas aeruginosa* ATCC 27853 | 40.9 ± 5.1 |
| *Kocuria rhizophila* ATCC 9341 | 96.6 ± 2.9 |
| *Bacillus subtilis* ATCC 6633 | 11.8 ± 3.7 |
| *Escherichia coli* ATCC 25992 | 15.7 ± 3.0 |
| *Bacilus cereus* ATCC 11778 | 14.9 ± 1.1 |
| *Staphylococcus aureus* ATCC 6538 | 5.9 ± 1.1 |

Atraric acid-containing lichens including *Evernia prunastri*, *Parmelia sulcata*, *Flavorparmelia caperata,* and *Hypogymnia physodes* were previously reported to possess antibacterial activity against *S. aureus* with MIC values of 156, 313, 125, and 39.1 µg/mL, respectively.[21] Anti-candidiasis activity of atraric acid isolated from *Pseudevernia furfuracea* (L.) Zopf was previously evaluated against *Candida krusei* and *Candida dubliniensis* with inhibition zones value of 25 and 23 mm, respectively.[22] *Parmotrema rampoddense* was reported to contain atraric acid in which antibacterial activity evaluation against Methicillin-Susceptible *Staphylococcus aureus* (MSSA) ATCC 25923 showed significant activity with a minimum inhibition concentration (MIC) value of 19.2 µg/mL.[23] Atraric acid containing crude extract of *Flavoparmelia caperata* lichen was reported to have bactericidal activity against *E. coli* with inhibitory zones 15.3 mm and MIC 15 mg/mL.[24] Previous pharmacological studies of atraric acid against non-infective diseases demonstrated that atraric acid isolated from the lichen *Heterodermia hypoleuca* regulated inflammation by inducing pro-inflammatory cytokine, nitric oxide, prostaglandin E2, induced nitric oxide synthase, and cyclooxygenase-2 enzyme expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.[25] In addition, natural atraric acid was able to act as an antagonist of androgen receptors by inhibiting the growth of prostate cancer cells.[26] Other reports stated the presence of methyl *β*-orcinol carboxylate (atraric acid), atranorin, lecanorol, and salazinic acid in *Parmotrema tinctorum* enhance absorption of UV radiation which is categorized as UVB blockers.[27]

## CONCLUSION

This phytochemical study revealed the first report on the major constituent of *P. aciculare* as methyl 2,4-dihydroxy-3,6-dimethylbenzoate. The crude extract possessed significant antibacterial activity against *K. rhizophita* at a relatively low concentration. This study contributed to further extensive bioprospecting studies on understudied Indonesian lichens.

## ACKNOWLEDGEMENT

The research was conducted with support from the University of Jember and the University of Wollongong.

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