The phytochemical investigation from \(n\)-hexane extract of the lichen *Roccella montagnei*

**Duong Thuc Huy\(^1\), Nguyen Thi Hoai Thu\(^2,\,*\)**

**ABSTRACT**

**Introduction:** *Roccella montagnei* is widely distributed in subtropical regions. As the continuous study on the hexane extract of *Roccella montagnei* lichen, the isolation and structural determination of five compounds were addressed. **Method:** The crude extract was obtained from the dried lichen powder’s extraction at room temperature. The \(n\)-hexane, \(n\)-hexane-ethyl acetate, and ethyl acetate extracts were obtained by the liquid-liquid partition method. The organic compounds were isolated from \(n\)-hexane extract by silica gel and Sephadex LH-20 column chromatography. Their chemical structures were identified by the NMR and HR-ESI-MS data analysis and the comparison of their NMR data with the published data. **Results:** Five compounds were isolated and chemically structural identified, consisting of 3,5-deoxy-7\(\alpha\)-methoxy-stigmast-5-ene (1), sekikaic acid (2), lichenxanthone (3), (+)-6,8-dihydroxy-3-propyl-3,4-dihydroisocoumarin (4), and ar-turmerone (5). **Conclusion:** To the best of our knowledge, except 3 which was reported from this species for the first time, four isolated compounds left did not known to be present in *Roccella* genus before. **Key words:** *Roccella montagnei*, lichen, sterol, aromatic compound

**INTRODUCTION**

*Rocella* genus distributes in the subtropical regions, Mediterranean and mainly in the Northern hemisphere\(^1\). *Rocella montagnei* species is commonly found along the Coromandel coast and in the mangrove forest in Indian and in the South of Vietnam\(^2\). The previously chemical investigations on this species showed the presence of depside, depsidone, quinone, sterol, usnic acid derivatives, and phenolic compounds\(^3–5\). The bioactive assessments on extracts and isolated compounds from this species revealed anti-inflammatory, antimicrobial, anti-arthritis, antioxidant, and anticancer activities\(^2,4,6,7\). The phytochemical investigation on this species was further studied on \(n\)-hexane extract led to the isolation and structural elucidation of 5 compounds.

**MATERIALS AND METHODS**

**General experimental procedures**

NMR spectra were recorded on Bruker Avance at 500 MHz for \(^1\)H-NMR and 125 MHz for \(^{13}\)C-NMR or on Bruker Avance III at 400 MHz for \(^1\)H-NMR and 100 MHz for \(^{13}\)C-NMR. In addition, the HR-ESI-MS spectra were acquired on a Bruker MicroOTOF-Q 10187. The NMR and MS spectra were done at the Center Analysis Laboratory of the University of Science, Vietnam National University (VNU)-Ho Chi Minh City and the Institute of Drug Quality Control Ho Chi Minh city.

**Plant material**

The lichen *Roccella montagnei* was collected at Tuy Phong District, Binh Thuan Province, Vietnam, in June 2018. The scientific name of the lichen was authenticated by Dr. Holger Thüs, Life Science Department, The Natural History Museum, Cromwell Road, SW7 5BD London, England, UK. In addition, a voucher specimen (No US–B024) was deposited in the Herbarium of Department of Organic Chemistry, Faculty of Chemistry, University of Science, VNU HCM.

**Extraction and isolation**

The fresh lichen *Roccella montagnei* (7.5 kg) was cleaned, air-dried, and ground into powder. Then, 876.8 g of crude extract was prepared using the above lichen powder in ethanol (3x10L) at room temperature. Next, this crude extract was applied to liquid-liquid partition step with \(n\)-hexane (100%), \(n\)-hexane:ethyl acetate (1:1, \(v/v\)), and ethyl acetate (100%) in turn to afford extracts, including \(n\)-hexane (H, 200.9 g), \(n\)-hexane:ethyl acetate (1:1) (H:EA, 225.5 g), and ethyl acetate (EA, 313.6 g). The \(n\)-hexane extract was subjected to silica gel column chromatography which was eluted with \(n\)-hexane, ethyl acetate, and ethyl acetate.
hexane: ethyl acetate (13:1) to give seven subfractions (coded, PH1-PH7). PH2 (43.7 g) was applied to Sephadex-LH20, eluted with methanol to obtain five subfractions (coded, 2.1-2.5). Then, two compounds, 1 (10 mg) and 5 (8.7 mg) were isolated from sub-fraction 2.2 (4.5 g) by the silica gel column chromatography method, eluted with the solvent system of n-hexane:chloroform: ethyl acetate (15:1:1). Next, PH4 (6.7 g) was separated into four sub-fractions 4.1 to 4.1 by the Sephadex-LH20 column chromatography, eluted with methanol. Afterward, sub-fractions 4.3 (2.4 g) was subjected to the C-18 reversed-phase silica gel column chromatography, eluted with the solvent system of Me: H$_2$O (15:1) to obtain 3 (11 mg) and 4 (7 mg). The same procedure was applied to sub-fraction PH7 (50.8 g) to afford 2 (9.5 mg).

**RESULTS**

From the n-hexane extract of the lichen *Roccella montagnei*, collected at Binh Thuân Province, five compounds, 1 (10 mg), 2 (9.5 mg), 3 (11 mg), 4 (7 mg), and 5 (8.7 mg), were isolated. Their physical properties and spectroscopic data were performed as follows.

3β-Hydroxy-7α-methoxystigmast-5-ene (1): Colorless powder. The $^1$H-NMR (CDCl$_3$): $\delta_H$ 3.62 (tt, 5.0, 11.0 Hz, H-3), 2.36 (ddd, 1.8, 5.0, 13.0, H-4a), 2.30 (ddt, 1.5, 11.0, 13.0, H-4b), 5.74 (dd, 2.0, 5.0, H-6), 3.29 (brt, 4.0, H-7), 1.96 (dt, 3.5, 12.5, H-12), 0.66 (s, H-18), 0.98 (s, H-19), 0.92 (d, 6.5 Hz, H-21), 0.81 (d, 7.0 Hz, H-26), 0.83 (d, 6.5 Hz, H-27), 0.86 (t, 7.5 Hz, H-29), 3.35 (s, -OCH$_3$). The $^{13}$C-NMR (CDCl$_3$): $\delta_C$ 36.9 (C-1), 31.6 (C-2), 71.6 (C-3), 42.5 (C-4), 146.3 (C-5), 120.9 (C-6), 74.1 (C-7), 37.4 (C-8), 42.9 (C-9), 37.6 (C-10), 21.0 (C-11), 39.2 (C-12), 42.3 (C-13), 49.2 (C-14), 24.4 (C-15), 28.4 (C-16), 55.9 (C-17), 11.6 (C-18), 18.4 (C-19), 36.3 (C-20), 19.0 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.4 (C-25), 19.2 (C-26), 20.0 (C-27), 23.3 (C-28), 12.2 (C-29), 56.9 (-OCH$_3$).

Sekikaic acid (2): Colorless solid. The $^1$H-NMR (CDCl$_3$): $\delta_H$ 6.38 (s, H-3), 6.38 (s, H-5), 2.99 (m, H-8), 1.74 (m, H-9), 0.94 (t, 7.2 Hz, H-10), 3.83 (s, 4-OCH$_3$), 6.43 (s, H-5′), 2.97 (m, H-8′), 1.64 (m, H-9′), 1.00 (t, 6.8 Hz, H-10′), 3.89 (s, 4′-OCH$_3$). The $^{13}$C-NMR (Acetone-d$_6$): $\delta_C$ 105.5 (C-1), 165.8 (C-2), 99.7 (C-3), 165.4 (C-4), 111.4 (C-5), 149.0 (C-6), 169.3 (C-7), 39.2 (C-8), 26.1 (C-9), 14.6 (C-10), 55.9 (4-OCH$_3$), 107.0 (C-1′), 157.3 (C-2′), 125.6 (C-3′), 156.2 (C-4′), 106.7 (C-5′), 146.8 (C-6′), 174.2 (C-7′), 39.2 (C-8′), 25.7 (C-9′), 14.6 (C-10′), 56.5 (4′-OCH$_3$). Selected HMBC correlations: see Figure 2.

**DISCUSSION**

Compound 1 was isolated as a colorless powder. The $^1$H-NMR spectrum showed an olefinic methine proton signal at $\delta_H$ 5.74 (dd, 2.0, 5.0, H-6), two oxygen-methyl groups protons at $\delta_H$ 3.62 (tt, 5.0, 11.0 Hz, H-3) and 3.29 (brt, 4.0, H-7), a methyl proton signal at $\delta_H$ 3.35 (s, -OCH$_3$) and the rest proton signals resonating at high magnetic field including two singlets ($\delta_H$ 0.66 and 0.98), three doublets ($\delta_H$ 0.92, 0.81, and 0.83) and a triplet ($\delta_H$ 0.86) methyl signals which corresponded to the stigmastane skeleton. It corresponded to the presence of a methoxy carbon and 29 carbons of the stigmastane. Two olefinic carbon signals resonated at $\delta_C$ 146.3 and 120.9 were a quaternary olefinic carbon (=C=, C-5) and a methine carbon (=CH -, C-6), respectively, in stigmast-5-ene as usual. The proton H-3 showed the large coupling constant value of 11.0 Hz with both axial protons H-2 and H-4, which approved the $\beta$-configuration of the hydroxy group at C-3. Pettit and co-worker$^3$ reported that the 7-methoxy-stigmast-5-ene compounds displayed a large coupling constant of 8.2 Hz of proton H-7 and a small coupling constant of 4.8 Hz of proton H-7$^\beta$. The compound 1 revealed the signal at $\delta_H$ 3.29 (brt, 4.0, H-7), demonstrating the $\alpha$-configuration of the 7-methoxy group. Based on the above analysis and the good compatibly NMR data with those published.
Compound 2 was isolated as a colorless solid. The $^1$H-NMR spectrum displayed aromatic proton signals at $\delta_H$ 6.38 (2H, s, H-3/H-5) of a tetra-substituted benzene ring and 6.43 (s, H-5') of a penta-substituted one. Two methoxy proton signals were observed at $\delta_H$ 3.83 (s, 4-OCH$_3$), 3.89 (s, 4'-OCH$_3$). At the higher magnetic field, it showed signals of two n-propyl moieties at $\delta_H$ [2.99 (m, H-8), 1.74 (m, H-9)], 0.94 (t, 7.2 Hz, H-10)] and [2.97 (m, H-8'), 1.64 (m, H-9'), 1.00 (t, 6.8 Hz, H-10')] which were further identified by the HMBC cross-peaks as shown in Figure 2.

The $^{13}$C-NMR spectrum showed 22 signals consisting of twelve signals of two benzene rings from 99.7 to 165.8 ppm, two carboxyl carbons at $\delta_C$ 169.3 (C-7) and 174.2 (C-7'), two methoxy carbons at $\delta_C$ 55.9 (4-OCH$_3$) and 56.5 (4'-OCH$_3$), and six final carbon signals from 14.6 to 39.2 ppm belonging to two n-propyl chains. The position of two methoxys were C-4 and C-4', which were confirmed by HMBC correlations of the methoxy protons to carbons at $\delta_C$ 165.4 (C-4) and 156.2 (C-4'). The HMBC correlations of H-8 to carbon C-1, C-5, and C-6, of H-8' to carbons C-1', C-5', and C-6' determined the positions of two n-propyl groups at C-6 and C-6'. All other HMBC correlations confirmed the chemical structure of 2. Additionally, the comparison of NMR data of 2 with the published data showed good compatibility; therefore, 2 was assigned as sekikaic acid.

Compound 3 was isolated as a white crystal. The $^1$H-NMR spectrum showed a signal at $\delta_H$ 13.39 (s, 1-OH) of the proton of a phenolic hydroxy group which was chelated to an ortho-carbonyl group, two meta-doublet signals which had the multiplet skewing effect together at $\delta_H$ 6.30 (d, 2.0 Hz, H-2), 6.34 (d, 2.4 Hz, H-4) of a tetra-substituted benzene ring, two other meta-doublet signals $\delta_H$ 6.69 (d, 2.4 Hz, H-5), 6.67 (d, 2.0 Hz, H-7) of the second tetra-substituted benzene ring. The moderate magnetic zone revealed signals of two methoxy groups at $\delta_H$ 3.87 (s, 3-OCH$_3$) and 3.90 (s, 6-OCH$_3$). The final proton signal at $\delta_H$ 2.85 (s, H-9) was belonged to a methyl group connected to a benzene ring. Besides, the NMR data of 3 showed highly relevant to the published data, 3 was thus determined as lichenxanthone.

Compound 4 was isolated as a colorless needle crystal. The HR-ESI-MS of 4 showed the$sodiated$ ion peak at m/z 245.0794 [M+Na]$^+$ (calcd. for C$_{12}$H$_{14}$O$_7$Na, 245.0790) which deduced its molecular formula to be C$_{12}$H$_{14}$O$_7$. The $^1$H-NMR spectrum displayed a singlet proton signal at $\delta_H$ 11.20 (s, 8-OH) of a phenolic hydroxy group which was chelated to an ortho-carbonyl group. Two broad-singlet proton signals at 6.31 (brs, H-5) and 6.21 (brs, H-7) suggested the presence of a 1,2,3,5-tetra-substituted benzene ring. A very broad-singlet signal integrated one proton at $\delta_H$ 6.00 (brs, 6-OH) was deduced to a hydroxy group which did not exist in the intramolecular hydrogen bond. There was a multiplet signal at $\delta_H$ 4.53 (H) of an oxygenated methine group in the magnetic zone of protons on the carbon attached to the single-bonded oxygen. Signals of two non-equivalent protons of a methylene group deshielded at $\delta_H$ 2.82 (dd, 4.4, 16.4 Hz, H-4a) and 2.88 (dd, 10.4, 16.0 Hz, H-4b) due to the attachment to sp$^2$ quaternary carbon and a chiral methine group. At highest magnetic field, the observation of a triplet signal, integrated three protons at 0.97 (t, 7.2 Hz, H-3') belonged a methyl group which was adjacent one methylene group. Four remaining protons at $\delta_H$ 1.68 (1H, m, H-1'a), 1.88 (1H, dddd, 5.2, 7.6, 10.0, 13.6 Hz, H-1'b), 1.49 (1H, m, H-2'a), and 1.58 (1H, m, H-2'b) were deduced the presence of two methylene groups. Based on the above information and the good compatibility of its NMR data with those published in the literature, along with its deextrorotatory activity [a]$_D^{20}$ = + 170 (c 0.1, MeOH), 4 was determined as (+)-6,8-dihydroxy-3-propyl-3,4-dihydroisocoumarin.

Compound 5 was isolated as a yellow solid. The $^1$H-NMR spectrum of 5 displays a singlet proton signal integrated four-protons at $\delta_H$ 7.10 ( , H-3/H-7; H-4/H-6) para-disubstituted benzene ring, a singlet
Figure 2: Selected HMBC correlations of 2 and 5

CONCLUSION

From the n-hexane extract of the lichen Roccella montagnei, five compounds were isolated consisting of a sterol (3β-hydroxy-7α-methoxy-stigmast-5-ene), a depside (sekikaic acid), a xanthone (lichenxanthone), a coumarin derivative ((+)-6,8-dihydroxy-3-propyl-3,4-dihydroisocoumarin), and an ar-turmerone (5). Their chemical structures were elucidated by NMR, MS spectroscopic data analysis, optical rotations, and the comparison to the published data. Four isolated compounds 1, 2, 4, and 5 were reported to presence in the Roccella genus for the first time while 3 had been already isolated from other species of this genus. The ongoing studies on this species are in progress.

ABBREVIATIONS

HR-ESI-MS: High resolution-Electrospray ionization-Mass spectrometry

1H-NMR: Proton nuclear magnetic resonance

13C-NMR: Carbon-13 nuclear magnetic resonance

HSQC: Heteronuclear single quantum coherence

HMBC: Heteronuclear multiple bond correlation

s: singlet

brs: broad singlet

d: doublet

t: triplet

m: multiplet

COMPETING INTEREST

The authors declare no competing financial interest.

AUTHORS’ CONTRIBUTION

Duong T.H contributed to conducting experiments, acquisition of data, and interpretation of data. Nguyen T. H. T interpreted NMR and MS data as well as gave final approval of the manuscript to be submitted.
ACKNOWLEDGMENT
This research was supported by NAFOSTED under grant number 104.01-2019.45.

REFERENCES
1. Prashanth SR, Bharath P, Valarmathi R, Balaji P, Parida A, Hariharan GN. Species status and relationship between Roccella montagnei and Roccella belangeriana using DNA sequence data of nuclear ribosomal internal transcribed spacer region. Journal of Plant Biochemistry & Biotechnology. 2008; 17(1):91-94; Available from: https://doi.org/10.1007/BF03263267.

2. Balaji P, Bharath P, Satyan RS, Hariharan GN. In vitro antimicrobial activity of Roccella montagnei thallus extracts. Journal of Tropical Medicinal Plants. 2006; 7(2):169-173;

3. Duong TH, Huynh BLC, Chavasiri W, Chollet-Krugler M, Nguyen VK, Nguyen THT, Hansen PE, Le Pogam P, Thüs H, Boustie J, Nguyen KPP. New erythritol derivatives from the fertile form of Roccella montagnei. Phytochemistry. 2017; 137:156-164; PMID: 28222890. Available from: https://doi.org/10.1016/j.phytochem.2017.02.012.

4. Tatipamula VB, Vedula, GS, Sastry AVS. Chemical and pharmacological evaluation of manglicolous lichen Roccella montagnei Bel em. D. D. Awasthi. Future Journal of Pharmaceutical Sciences. 2019; 5:8; Available from: https://doi.org/10.1248/cpb.c12-00739.

5. Mallavadhani UV, Sudhakar AVS. Roccellatol, a new β-orcinol based metabolite from the lichen Roccella montagnei. Natural Product Research. 2017; 32:268-274; PMID: 28726494. Available from: https://doi.org/10.1080/14787419.2017.1353508.

6. Vijayakumar CS, Viswanathan S, Reddy MK, Parvathavarthini S, Kundu AB, Sukumar E. Anti-inflammatory activity of (+)-usnic acid. Fitoterapia. 2000; 71:564-566; Available from: https://doi.org/10.1016/S0367-326X(00)00209-4.

7. Mishra T, Shukla S, Meena S, Singh R, Pal M, Upeti KD, Datta D. Isolation and identification of cytotoxic compounds from a fruticose lichen Roccella montagnei and its in silico docking study against CDK-10. Revista Brasileira de Farmacognosia. 2017; 27(6); Available from: https://doi.org/10.1016/j.rbpj.2017.07.006.

8. Pettit GR, Numata A, Cragg GM, Herald DL, Takada T, Iwamoto C, Riesen R, Schmidt JM, Doubek DL, Goswami A. Isolation and structures of schleicherastatins 1-7 and schleicheols 1 and 2 from the Teak forest medicinal tree Schleicheria oleosa. Journal of Natural Product. 2000; 63:72-78; PMID: 10650082. Available from: https://doi.org/10.1021/np990346r.

9. Lai D, Odimegwu DC, Grunwald T, Esmione C, Proksch P. Phenolic compounds with in vitro activity against respiratory syncytial virus from the Nigerian lichen Ramalina farinacea. Planta Medica. 2013; 79:1440-1446; PMID: 23970423. Available from: https://doi.org/10.1055/s-0033-1350711.

10. Brandão LFG, Alcantara GB, Matos M de FC, Bogo D, Freitas D dos S, Oyama NM, Honda NK. Cytotoxic evaluation of pheno- lic compounds from lichens against melanoma cells. Chemical and Pharmaceutical Bulletin. 2013; 61(2):176-183; PMID: 23207680. Available from: https://doi.org/10.1095/chb.12-00739.

11. Xie T, Zheng C, Chen K, He H, Gao S. Asymmetric total synthesis of the complex polycyclic xanthone FD-594. A Journal of the German Chemical Society. 2020; 59(11):4360-4364; PMID: 31943607. Available from: https://doi.org/10.1002/anie.201915787.

12. Dhingra OD, Jham GN, Barcelos RC, Mendonça FA, Ghiviriga I. Isolation and identification of the principal fungitoxic component of Turmeric essential oil. Journal of Essential Oil Research. 2007; 19:387-391; Available from: https://doi.org/10.1080/10412905.2007.9699312.

13. Hucklebroich J, Klein R, Neumaier B, Graf R, Fink GR, Schroeter M, Rueger MA. Aromatic-turmerone induces neural stem cell proliferation in vitro and in vivo. Stem Cell Research & Therapy. 2014; 5:100; PMID: 25928248. Available from: https://doi.org/10.1186/s13287-014-0003-0.