Revista Brasileira de Farmacognosia 27 (2017) 724–728

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Isolation and identification of cytotoxic compounds from a fruticose lichen *Roccella montagnei*, and it's *in silico* docking study against CDK-10

Tripti Mishra[a], Shipra Shukla[a], Sanjeev Meena[b], Ruchi Singh[c], Mahesh Pal[a,*], Dalip Kumar Upreti[d], Dipak Datta[b]

[a] *Phytochemistry Division, CSIR-National Botanical Research Institute, Lucknow 226 001, India*
[b] *Biochemistry Division, CSIR-Central Drug Research Institute, Lucknow 226031, India*
[c] *Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India*
[d] *Plant Diversity, Systematics and Herbarium Division, CSIR-National Botanical Research Institute, Lucknow 226001, India*

ARTICLE INFO

Article history:
Received 6 February 2017
Accepted 7 July 2017
Available online 7 September 2017

Keywords:
Docking study
Roccellic acid
Everninic acid
Cytotoxic activity

ABSTRACT

Roccella montagnei Bél. belongs to lichen family Roccelleceae growing luxuriantly along the coastal regions of India. As *Roccella* has been shown to be bioactive, we prepared methanolic extract and assessed its anticancer potential. The methanolic extract showed significant *in vitro* cytotoxic activity against four human cancer cell lines such as colon (DLD-1, SW-620), breast (MCF-7), head and neck (FaDu). This prompted us to isolate bioactive compounds through column chromatography. Two compounds roccellic acid and everninic acid have been isolated, out of which everninic acid is reported for the first time. Both the compounds have been tested for *in vitro* cytotoxic activity in which roccellic acid showed strong anticancer activity as compared to the everninic acid. Cyclin Dependent Kinase (CDK-10) contributes to proliferation of cancer cells, and aberrant activity of these kinases has been reported in a wide variety of human cancers. These kinases therefore constitute biomarkers of proliferation and attractive pharmacological targets for development of anticancer therapeutics. Therefore both the isolated compounds were tested for *in silico* molecular docking study against Cyclin Dependent Kinase isomer enzyme to support the cytotoxic activity.

Introduction

Lichen is a symbiotic self-supporting mutualism shows a wide range of habitats throughout the world. It is a stable organism between mycobiont and photobiont. (Hawksworth and Honegger, 1994) They found growing on rocks, bricks, soil, rotting wood *etc.* Lichens are a potential source of different biological activity as antitubercular (Marshak and Kuschner, 1950), anticancer (Williams et al., 1998), anti-HIV (Huneck and Yoshimura, 1996) antipyretic analgesic (Müller, 2001).

The lichen species *Roccella montagnei* Bél. belongs to family Roccellaceae, found common as epiphytes along the Coromandel Coast, Tamil Nadu, India and it is abundant in Pichavaram mangrove forests. It is a fruticose growth form (Tehler et al., 2004). *R. montagnei* is a rich source of so many secondary metabolites as orcinol, montagnetol, β-carotene, β-sitosterol, erythritol, roccellic acid, lecanoric acid and methyl orsellinate *etc.* (Mittal et al., 1952). A previous report confirms the biological importance of *R. montagnei* for its antimicrobial activity (Balaji et al., 2006), insecticidal activity (Nanayakkara et al., 2010) and anti-inflammatory activity (Cetin et al., 2008).

The present work deals with the primary screening of cytotoxic activity of methanolic extract, isolation of bioactive molecules, structure elucidation of isolated compounds and identification of compound responsible for cytotoxic activity of *R. montagnei*.

Materials and methods

Lichen material

In the present study, lichen materials (*Roccella montagnei* Bél., Roccellaceae) were collected from Kovalam Sea shore Thirvananthpuram, Kerala, India grown over *Cocoes nucifera* barks in the month of August 2013 and were authenticated by Taxonomy Division, CSIR

* Corresponding author.
E-mail: m.pal@nbri.res.in (M. Pal).

https://doi.org/10.1016/j.bjp.2017.07.006

– National Botanical Research Institute Lucknow, India. Herbarium specimens (12634) were prepared and deposited at the Herbarium of the Institute.

Extraction

Dried lichen material (100 g) were milled into powder and then extracted with methanol (2.5 l) in an extractor for 36 h. The extract was evaporated in a rotatory evaporator and dried by vacuum pump to afford the 7.5 g of methanolic extract.

Compound isolation

Methanolic fraction (5 g) was subjected to chromatography on silica gel (60–120 mesh) eluted with a step wise gradient of hexane-ethyl acetate (9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3) by volume to afford a total of 500 fractions of 50 ml each. Column fractions were analyzed by TLC, and fractions with similar TLC patterns were combined to give five major sub column fractions. Column fraction-1 was further purified to give 21.5 mg of everninic acid (21.5 mg) (**1**) white crystalline compound (Yusof et al., 2015). Fraction 2 was further purified to give 16 mg of white powdered **(2)** Roccellic acid (Huneck and Yoshimura, 1996).

Cytotoxic activity

Cell culture and sample preparation

Methanolic extract along with isolated compound of *R. montagnei* were tested for *in vitro* cytotoxic activity against five cancer cell lines. The human cancer cell lines such as colon (DLD-1) and breast (MCF-7) were maintained in RPMI-1640 medium, whereas head and neck (FaDu) and colon cell lines (SW-620) in DMEM medium. The test samples/molecules weighed in micro-centrifuge tubes and stock solutions of 100 mg/ml were prepared by dissolving the samples in DMSO. Stock solutions were stored at −20 °C. A working solution of 100 μg/ml was prepared by diluting the stock solution in culture medium (RPMI-1640 with 5% FBS) prior to the assay.

Cytotoxicity assay (SRB assay)

The standard colorimetric SRB assay was used for the measurement of cell cytotoxicity (Krishna et al., 2014; Mishra et al., 2016). In brief, 10,000–30,000 cells depending on the doubling time of each cell type were seeded to each well of 96-well plate in 5% serum containing growth medium and incubated overnight to allow for cell attachment. Cells were then treated with the test sample (100 μl) to give a final concentration of 100 μg/ml and duplicate wells were included. Untreated cells receiving the same volume of vehicle containing medium served as control. After 48 h of exposure, cells were fixed with ice-cold 50% TCA, stained with 0.4% (w/v) SRB in 1% acetic acid, washed and air dried. Bound dye was dissolved in 10 mM Tris base and absorbance was measured at 510 nm on a plate reader (Epoch Microplate Reader, Biotek, USA). The cytotoxic effects of compounds were calculated as percentage inhibition in cell growth as per the formula.

$$\%\ \text{of cells killed} = 100 - \left\lfloor \frac{\text{MeanOD}_{\text{test}}}{\text{MeanOD}_{\text{control}}} \times 100 \right\rfloor$$

Molecular docking studies

Compound roccellic acid and everninic acid isolated from *R. montagnei* have been evaluated for cytotoxic activity and further as a supportive study these two compounds along with the standard drug doxorubicin have been evaluated for *in silico* molecular docking study. Therefore, in this study the compounds were selected as the ligand by using Homo sapiens Cyclin Dependent Kinase-10 (CDK-10 isomer) as the base enzyme. The protein encoded by this gene belongs to the CDK subfamily of the Ser/Thr protein kinase family. The CDK subfamily members are known to be essential for cell cycle progression. This kinase has been shown to play a role in cellular proliferation and its function is limited to cell cycle G2-M phase. Cyclin dependent kinases contributes to proliferation of cancer cells, and aberrant activity of these kinases has been reported in a wide variety of human cancers. These kinases therefore constitute biomarkers of proliferation and attractive pharmacological targets for development of anticancer therapeutics. (Peyressatre et al., 2015). The autodock 4.2 docking software was used to perform molecular docking simulation between CDK-10 and the compounds isolated from *R. montagnei* along with doxorubicin. Sequence has been obtained by NCBI and model of CDK-10 has been prepared from ITASSER server (Zhang, 2007). MGLTools-1.4.6 was used to prepare protein (protein.pdbqt) and to write grid parameter file (protein.gpf) and docking parameter file (ligand.dpf). Protein preparation includes: (i) removal of water and ions and extraction of co-crystallized ligand; (ii) addition of polar hydrogens; (iii) assignment of AD4 atom type; and finally (iv) assignment of Gasteiger charges. The grid maps representing the native ligand in the actual docking target site were calculated with autogrid4 with box dimension of 126 × 126 × 126 Å and spacing of 0.375 Å by taking the center of the ligand as the center of the grid. Docking of the ligand was done with default parameters.

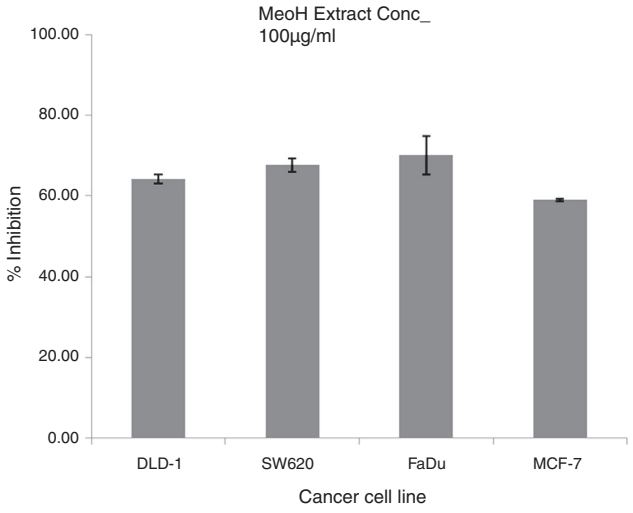


Fig. 1. Cytotoxic activity of the methanol extract (100 μg) against breast and colon cancer cell lines.

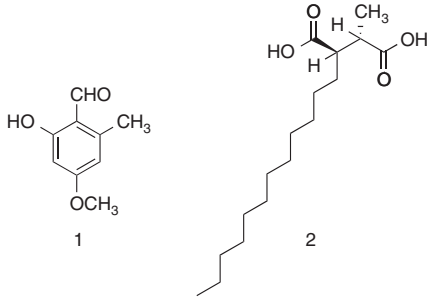
Result and discussion

Cytotoxic activity

Roccella montagnei thallus was extracted in methanol and methanolic extract was screened primarily for *in vitro* cytotoxic activity against five different human cancer cell lines such as colon (DLD-1, SW-620), breast (MCF-7), head and neck (FaDu). Methanolic extract were found to have 62.99%, 66.889% cell growth inhibition activity against colon cancer cell line *i.e.* DLD-1 & SW-620 respectively (Fig. 1). Moreover against head and neck (FaDu) cancer cell line it shows 64.05% cell growth Inhibition. Methanolic extract shows moderate cell growth inhibition against breast cancer cell line *i.e.* 56.55%. Methanolic extract has been selected to isolate pure molecules responsible for cytotoxic activity.

Isolation of pure compounds from methanolic extract of Roccella montagnei

The methanolic extract was column chromatographed over silica to obtain pure compounds. Isolated pure compounds were identified by means of spectroscopic analysis, and they were identified as everninic acid (**1**) and roccellic acid (**2**). Everninic acid (**1**) resulted as a white crystalline solid and its mass spectrum exhibited molecular ion peak at *m*/*z* 182, with the molecular formula $C_9H_{10}O_4$. UV spectroscopy signifies absorption bands at 550 nm, moreover IR spectra of compound **1** showed frequencies at 3390 cm^{-1} and 3400 cm^{-1} indicating the presence of aromatic hydroxyl group. MS spectra shows base peak at *m*/*z* 149 were attributed to orsellinic acid moiety. Proton NMR spectroscopy shows peak at 11.58 for hydroxyl group. Two signals at 6.19 and 6.20 attributed to aromatic proton methyl singlet at 2.438 for methyl group present at benzene ring. Signal at 3.93 denotes for methoxy group. The ^{13}C NMR spectra showed nine carbons, signal at δ173.559 showed presence of carboxylic group and signal at 52.344 shows presence of methoxy group which were the characteristic signals of the compound (**1**). Signal at 166.031, 163.803, 144.684 and 106.073 indicated quaternary carbons along with 101.867, 112.640 for tertiary carbon one signal at 24.271 shows methyl group presence (Yusof et al., 2015). All the spectroscopic details indicated that it is orsellinic acid-4-methylether (everninic acid) with molecular formula $C_9H_{10}O_4$. Roccellic acid (2) resulted as white amorphous solid and its mass spectrum shows molecular ion peak at *m*/*z* 300, with the molecular formula moreover IR spectra of compound **2** shows frequencies at 3000, 2900 corresponds to carboxylic group. MS spectra shows base peak at 283. Proton NMR spectroscopy shows peak at 0.88 (t, $-CH_3$), 1.17 (d, $-CH_3$), 1.284 (s, $-CH_2$). The ^{13}C NMR spectra showed nine carbons, Signal at δ178.988 and 178.244 showed presence of two carboxylic groups. Signal at 30.822 shows triplet and at 30.51 doublets and at 30.684 singlet peak. All the spectroscopic data indicated that the compound is Roccellic acid with molecular formula $C_{17}H_{32}O_4$ (Huneck and Yoshimura, 1996).



It is known that the everninic acid is already reported in *Cladonia multiformis*, however, to the best of our knowledge, the isolation and characterization of everninic acid from *R. montagnei* has not been reported earlier. Both the isolated compounds were identified on the basis of UV, IR and 1H NMR data and compared and validated with the existing literature.

Cytotoxic activity of isolated compound

Isolated compounds have been evaluated at 100 μg/ml dose were for *in vitro* cytotoxic activity against two cancer cell lines *i.e.* breast cancer (MCF-7, MDAMB-231) and colon cancer (DLD-1, SW-620) (Fig. 2). It is needed to mention here that the doses of pure compounds may vary at micromole level. The dose of 100 μg/ml of everninic acid and roccellic acid are 548.92 μM and 332.85 μM respectively as per calculation, thus the everninic acid shows lower cell growth inhibition percentage at higher dose at micromole scale and roccellic acid found to have significant cell growth inhibition

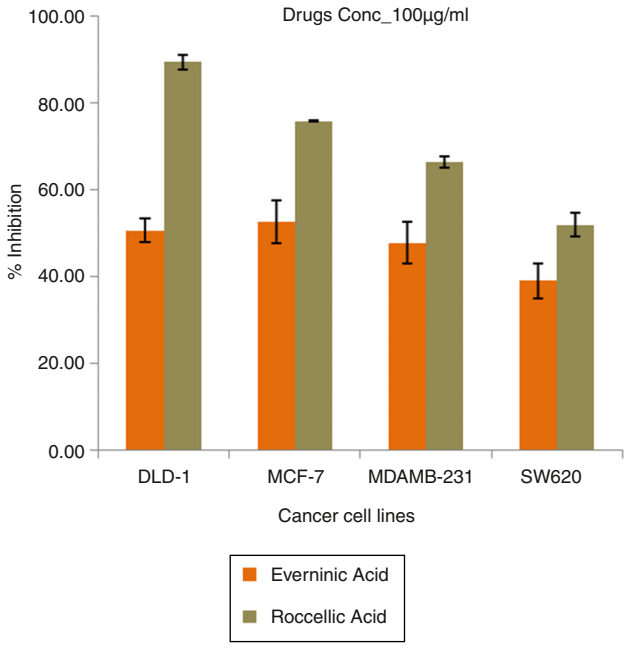


Fig. 2. Cytotoxic activity of isolated compounds against breast and colon cancer cell lines.

at 100 μg/ml (332.85 μM) dose against MCF-7 and DLD-1 *i.e.* 75.84 and 87.90%, respectively, however it was effective against MDAMB-231 with 65.30% cell growth inhibition. Out of isolated compounds roccellic acid was found to be cytotoxic against different cancer cell lines but shows most significant cytotoxic activity in colon cancer *i.e.* DLD-1. Thus IC_{50} of both the compounds have been calculated as per dose response curve against DLD-1 to compare the cytotoxic effects (Fig. 3). IC_{50} of roccellic acid was found 71.26 μg/ml (237.18 μM) whereas everninic acid shows IC_{50} value more than 100 μg/ml.

In-silico *comparative molecular docking studies of isolated compounds against CDK-10*

To investigate the effect of different compounds on cancer cell line we have chosen Cyclin Dependent Kinase-10 (CDK-10 isomer) of *Homo sapiens* as substrate because it is an important enzyme in the growth phase of the cancerous cells. Everninic acid and roccellic acid have chosen as ligands. Molecular docking is the simulations of binding of everninic acid and roccellic acid with active site of CDK10 was carried out using Autodock 4.2. From this study we can conclude about H-bond interactions with the active site residues. Everninic acid forms one H bond with the hydrogen of ARG71:HH11 along with the carbonyl oxygen of everninic acid LIG1:O. (Fig. 4) The estimated free binding energy of everninic acid is −6.65 kcal/mol with the estimated inhibition constant, Ki = 13.37 μM. Roccellic acid forms two hydrogen bonds, one with the hydrogen of roccellic acid along with nitrogen of ALA 187 and other is between oxygen of roccellic acid with hydrogen of ASN 343 HD22 (Fig. 5). Roccellic acid has shown estimated free energy of binding −6.75 kcal mol^{-1} estimated inhibition constant, Ki = 11.35 μM.

Conclusion

The aim of the present study was to isolate bioactive compound, which is responsible for significant cytotoxic activity of methanolic extract of *R. montagnei*. Two compound everninic acid and roccellic acid has been isolated from methanolic extract of *R. montagnei* and confirmed by spectroscopic techniques. To the best of our

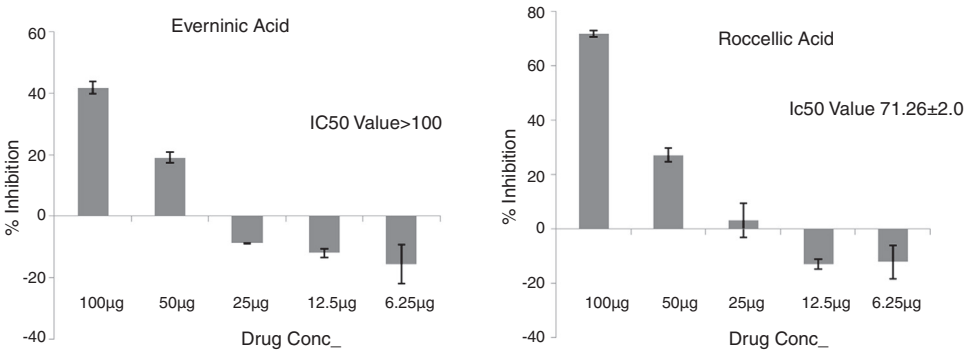


Fig. 3. Dose response study of everninic acid and roccellic acid against colon cancer cell line (DLD-1).

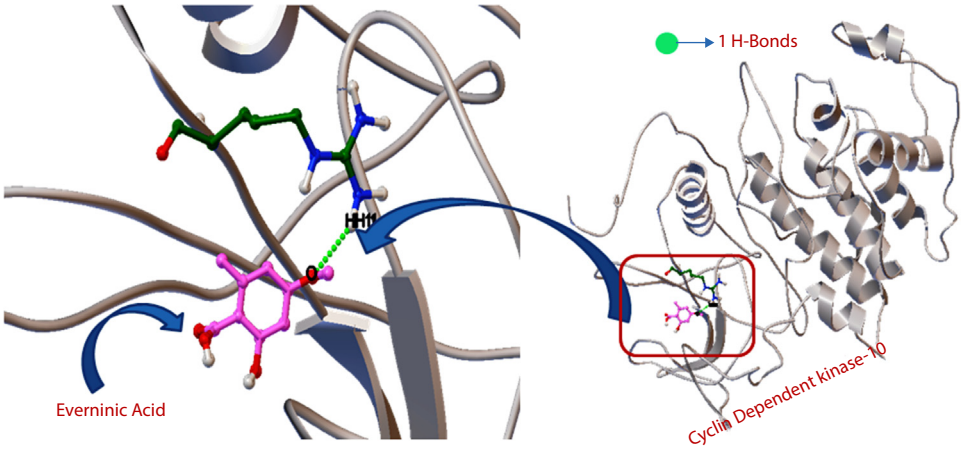


Fig. 4. Molecular docking study of everninic acid against cyclin dependent kinase-10.

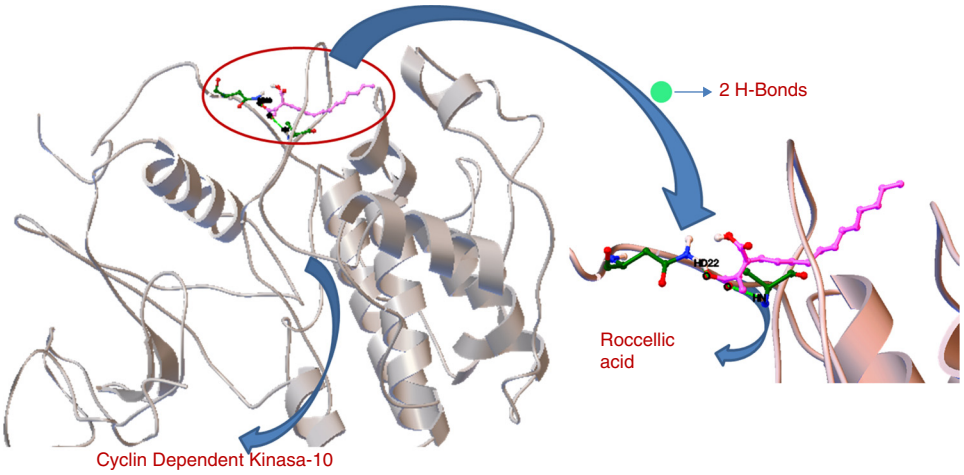


Fig. 5. Molecular docking study of roccellic acid against cyclin dependent kinase-10.

knowledge everninic acid is reported for the first time in *R. montagnei*. It is believed that majority of compounds originated from fungal component. Probably everninic acid might be originated from fungal component of *R. montagnei* as it is already reported from *Cladonia multiformis* (Yusof et al., 2015). Both the compounds have been evaluated against human cancer cell lines. At 100 µg roccellic acid shows significant activity against breast and colon cancer cell lines. Roccellic was found to have significant cell growth inhibition against DLD-1 with IC_{50} value 71.26 µg/ml as per dose response curve study. It is also supported by *in silico* molecular docking study which showed that everninic acid form one and roccellic acid showed two hydrogen bonding with free binding energy −6.65 kcal/mol and −6.75 kcal/mol respectively. Although roccellic acid form two hydrogen bond interaction but free binding energy of roccellic acid found to have lesser than everninic acid thus it is confirmed that roccellic acid found to have better cytotoxic activity over everninic acid.

Finally it can be concluded that cytotoxic activity of *R. montagnei* thallus might be due to presence of roccellic acid, but we cannot say that roccellic acid is solely responsible for its anticancer activity there may be some other compound which is responsible for their anticancer activity thus future prospects of the study is to identify

some other novel compound responsible for significant cytotoxic activity of *R. montagnei*.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Authors' contributions

Conceived and design the experiments: MP DD DKU. Performed the experiments: TM SS, SM, and RS. Analyzed the data: MP DD. Contributed reagents/material/analysis tools: DD DKU. Wrote the paper: MP TM SS SM.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors are thankful to the Director, CSIR-National Botanical Research Institute, Lucknow, India for facilities and encouragements. The financial support received from the Council of Scientific and Industrial Research, New Delhi under the project 'Bioprospection of Plant Resources and Other Natural Products (BSC-0106)' is duly acknowledged.

References

Balaji, P., Bharath, P., Satyan, R., Hariharan, G., 2006. *In vitro* antimicrobial activity of *Roccella montagnei* thallus extracts. J. Trop. Med. Plant 7, 169–173.

Cetin, H., Tufan-Cetin, O., Turk, A.O., Tay, T., Candan, M., Yanikoglu, A., Sumbul, H., 2008. Insecticidal activity of major lichen compounds, (−)-and (+)-usnic acid, against the larvae of house mosquito, *Culex pipiens* L. Parasitol. Res. 102, 1277–1279.

Hawksworth, D., Honegger, R., 1994. The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. In: Williams, M.A.J. (Ed.), Plant Galls. Organisms, Interactions, Populations, The Systematics Association Special Volume, vol. 49. Clarendon Press, Oxford, pp. 77–98.

Huneck, S., Yoshimura, I., 1996. Identification of Lichen Substances. Springer, Berlin.

Krishna, S., Singh, D.K., Meena, S., Datta, D., Siddiqi, M.I., Banerjee, D., 2014. Pharmacophore-based screening and identification of novel human ligase I inhibitors with potential anticancer activity. J. Chem. Inf. Model. 54, 781–792.

Marshak, A., Kuschner, M., 1950. The action of streptomycin and usnic acid on the development of tuberculosis in guinea pigs. Public Health Rep. (1896–1970), 131–144.

Mishra, T., Pal, M., Meena, S., Datta, D., Dixit, P., Kumar, A., Meena, B., Rana, T., Upreti, D., 2016. Composition and *in vitro* cytotoxic activities of essential oil of *Hedychium spicatum* from different geographical regions of western Himalaya by principal components analysis. Nat. Prod. Res. 30, 1224–1227.

Mittal, O., Neelakantan, S., Seshaadri, T., 1952. Chemical investigation of Indian lichens. XIV. Chemical components of *Ramalina calicaris* and *Ramalina sinensis*. J. Sci. Ind. Res. India B 11, 386–387.

Müller, K., 2001. Pharmaceutically relevant metabolites from lichens. Appl. Microbiol. Biotechnol. 56, 9–16.

Nanayakkara, C., Bombuwala, K., Kathirgamanathar, S., Adikaram, N., Wijesundara, D., Hariharan, G., Wolseleys, P., Karunaratne, V., 2010. Effect of some lichen extracts from Sri Lanka on larvae *Ofaedes aegypti* and the fungus *Cladosporium cladosporioides*. J. Natl. Sci. Found. Sri Lanka 33, 147–149.

Peyressatre, M., Prével, C., Pellerano, M., Morris, M.C., 2015. Targeting cyclin-dependent kinases in human cancers: from small molecules to peptide inhibitors. Cancers 7, 179–237.

Tehler, A., Dahlkild, Å., Eldenäs, P., Feige, G.B., 2004. The phylogeny and taxonomy of Macaronesian, European and Mediterranean *Roccella* (Roccellaceae, Arthoniales). Symb. Bot. Upsal. 34, 405–428.

Williams, D.E., Bombuwala, K., Lobkovsky, E., de Silva, E.D., Karunatne, V., Allen, T.M., Clardy, J., Andersen, R.J., 1998. Ambewelamides A and B, antineoplastic epidithiapiperazinediones isolated from the lichen *Usnea* sp. Tetrahedron Lett. 39, 9579–9582.

Yusof, H., Azahar, H., Din, L.B., Ibrahim, N., 2015. Chemical constituents of the lichens *Cladonia multiformis* and cryptothecia SP. Malay. J. Anal. Sci. 19, 930–934.

Zhang, Y., 2007. Template-based modeling and free modeling by I-TASSER in CASP7. Proteins: Struct. Funct. Bioinform. 69, 108–117.