Eco. Env. & Cons. 26 (3) : 2020; pp. (957-962)
Copyright@ EM International
ISSN 0971–765X

Short-term experiment on effects of sulfuric acid on atranorin concentration and upper surface structure in *Pyxine cocoes* (Sw.) Nyl

Chitsupang Kheawsalab [1, 2*], Wanaruk Saipunkaew[3], Somporn Chantara[4], Pitchaya Mungkornasawakul[4] and Nattakarn Sransupphasirigul[1, 2]

[1]Ph D Degree Program in Environmental Science, Environmental Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand
[2] Graduate School, Chiang Mai University, Chiang Mai, 50200, Thailand
[3] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand
[4] Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand

(Received 18 January, 2020; accepted 27 February, 2020)

ABSTRACT

Short-term experiment was performed to study effect of sulfuric acid solution in a lichen *Pyxine cocoes* (Sw.) Nyl. This study focuses on changes in atranorin content and morphology of upper surface structure in this species. High-Performance Liquid Chromatography with UV detector was used to determine concentrations of atranorin in *P. cocoes.* The results show that the highest average concentration of atranorin were found in a treatment with pH 3.0 which was 140.14ppm and the lowest concentration was found in a sample without any treatment which was 111.68ppm. There was no statistical correlation between treatments. Scanning Electron Microscope (SEM) was used to investigate upper surface and calcium oxalate crystal structure after samples were treated with acidic solution. SEM micrographs showed corrosive effects on calcium oxalate crystal surfaces that were cracks and many holes in all the treatments.

Key word : *Calcium oxalate crystal, HPLC, Pyxine cocoes, SEM, Sulfuric acid*

Introduction

In the recent times, fossil fuel is used in many processes and causes air pollutants such as acidic gases in the atmosphere. The United States Environmental Protection Agency (2008) provides a definition of the acid deposition from the atmosphere containing higher than normal amounts of nitric and sulfuric acids as wet and dry deposition. Wet deposition refers to acidic precipitation such as rain, fog, dew and snow whereas dry deposition refers to gases and dust particles that become acid. Acid deposition has various environmental and health effects on organisms. Sulfur dioxide (SO_2) is a harmful pollutant which can combine with water in the atmosphere that increase more acidic effects than normal. It can pass through to thallus quickly, causing unhealthy, bleached thallus, and/or death of lichen after absorbing those pollutions (Hauck, 2008; Zedda, 2009).

Epiphytic lichens are sensitive to air pollution. They can be used as air pollution indicators (Farinha *et al.,* 2009; Lackovicová *et al.,* 2013; Paoli *et al.,* 2015). They do not have any cuticle or wax to coat thallus structure that means water, gases, minerals, and air pollutants from the atmosphere are

absorbed directly in lichen thallus (Demirat *et al*., 2012). Air pollution can affect to lichen growth or living (Dzubaj *et al.*, 2008; Zedda, 2009) and their distributions (Svoboda *et al*., 2010; Jayalal *et al*., 2017). Each lichen species has a different chemical tolerance because they have some different upper surface structure, hydrophobicity, and secondary metabolite (Hauck *et al.*, 2008).

Lichen secondary metabolites are organic compound substances produced by mycobiont cell. Atranorin ($C_{19}H_{18}O_8$) is a common lichen secondary metabolite in depsides groups. It has many ecological benefits, for examples photoprotective activity (Lohézic-Le Dévéhat *et al.*, 2013), antioxidant activity (Kosanic *et al.*, 2014; Rajan *et al.*, 2016), etc. Bialoñska and Dayan (2005) found decreased in the levels of atranorin after lichens were transplanted near the chemical industrial areas. This compound was sensitive to heavy metal accumulation and acidic inorganic sulfur compounds (Bialoñska and Dayan, 2005). However, atranorin concentration was increased in some study sites which demonstrated a possible role of this compound in detoxification, or it might be a precursor of other phenolic compounds. They suggested that it could be used as biomarkers in environmental studies. Ohumra *et al.* (2009) found lower atranorin concentration in the polluted sites than in the clean site. They suggested that it might be related with the disintegration of the cortex and death of the photobionts. Kovácik *et al*. (2011) found increasing atranorin concentration in *Hypogymnia physodes* after treated with H_2SO_4 pH of 3.0. They demonstrated that only atranorin reacted with Folin-Ciocalteu reagent. Folin-Ciocalteu reagent test showed increasing insoluble phenols in acid rain-treated samples. They suggested that lichens seem to respond to acid rain by synthesis of specific metabolite.

Under acidic condition, upper surface structure of lichens can be changed. Upper part of some lichen is covered by a structure called pruina or calcium oxalate crystal. Calcium oxalate crystal in a lichen is the production between oxalic acid from primary substance of fungal cell and calcium (Purvis, 1984). The occurrence of calcium oxalates crystal was found on the outer surface of hyphae or accumulated on the upper cortex (Burford *et al.*, 2006). Smieja-Król *et al.* (2014) found the relationship between acidity and water content in lichen. In acid environment and less water were bound to the calcium oxalate and caused different crystal types forming in sites with various acidities or changes in crystal morphology. Garty *et al.* (2002) transplanted *Ramalina lacera* with in polluted areas and conducted *in situ* experiment under simulated acid rain. They found lacking calcium oxalate crystals in lichens which was transplanted in a polluted site and was sprayed with acid solution.

Pyxine cocoes (Sw.) Nyl. is a common foliose lichen in urban areas in northern Thailand. It can grow in large cities with high human activities (Saipunkaew *et al.*, 2007; Sransupphasirigul, 2012). None of previous studies had been reported on the effects of acid solution on the amount of atranorin in *P. cocoes* collected from northern part of Thailand. The aim of this study was to investigate the effects of acid solution exposure on atranorin concentration and to observe changes in upper surface structures of this species in a short-term laboratory experiment.

Materials and Methods

Lichen sampling

Lichen sampling were done on *Mangifera indica* L. (mango trees) in park area outside a center of Lampang city (18°18′N/99°28′E), Lampang province, Thailand. Thallus samples were randomly collected from a selected tree at height of 100-150 cm above the ground level. The selected samples were around 2.5 to 3 cm in diameter.

Acid solution treatment in the laboratory

Five lichen samples were separated from bark and cleaned up before further experiment. Clean samples were stored at 25 °C. Each thallus was cut into six pieces. These sample pieces were separated into five treatments and one for a blank sample. First treatment was soaked with deionized water (DI water), Second to fifth treatment were soaked with sulfuric acid solutions pH of 2.5, 3.0, 3.5 and 4.5 which was diluted with DI water, respectively and a blank sample which was not soaked with anything. Each sample was soaked for 30 min once a day under the room temperature for 15 days. After an experiment thallus was dried under 80 °C for 8 hr before analyzing with High-Performance Liquid Chromatography (HPLC) and Scanning Electron Microscope (SEM).

Analysis of atranorin in the thalli

Lichen extraction were extracted from 10 mg thalli

which suspended in 1 mL of 100% acetonitrile for 8 hr. Lichen extractions were analyzed by using High-Performance Liquid Chromatography (HPLC) model Agilent 1200 Series with UV detector set at a wavelength of 254 nm. The extractions were separated on a reversed phase C18, Inertsil ODS-3 column (5 µm, 150 × 4.6 mm, GL Sciences, Japan). The mobile phase consisted of methanol: deionize water: acetic acid (80:19.5:0.5) was used with a flow rate of 1.0 mL/min and the retention times of 25min. Benzoic acid was used as an internal standard. Atranorin concentration in each sample were calculated by a linearity curve of atranorin standard concentration curve which come from dividing atranorin peak area by benzoic acid peak area in known rang of atranorin standard. Percentage change of atranorin concentration in each treatment was calculated by comparing the concentration in each treatment with concentration from the blank sample.

Observation on changes in upper surface structure

Air-dried thallus tips of *P. cocoes* were coated with gold using a SPI-Module Sputter Coater and Vacuum Base with Etch Mode and Quartz Crystal Thickness Monitor (Structure Probe, Inc. West Chester, PA 19381-0656). Upper surface structure and crystals structure from lobes tips were examined in a LV-Scanning Electron Microscope: JSM 5910 LV (JEOL Ltd., Tokyo, Japan) running at 15 keV.

Data analysis

The result of average atranorin concentration in each treatment were compared by using one-way analysis of variance (ANOVA) and Tukey's test. Statistically differences were considered at $p \leq 0.05$.

Results and Discussion

Some lichen samples showed different appearances symptom. All samples in sulfuric acid treatment were bleached while there were no morphological changes in a group which was treated with DI water and on blank sample. HPLC chromatogram in Fig 1 shows two peak areas; first was benzoic acid, second one was atranorin. In this study benzoic acid used as an internal standard showed peak at 2.5 min and atranorin showed peak at 25.2 min.

Atranorin concentration was calculated by comparing retention time between *P. cocoes* extraction and atranorin standard extraction (Fig 2). Table 1 shows average atranorin concentration from all the treatments. Atranorin concentration in treatment pH 3 was the highest (140±21.90 ppm) and the lowest (111.68±26.61 ppm) was in blank samples which was not treated.

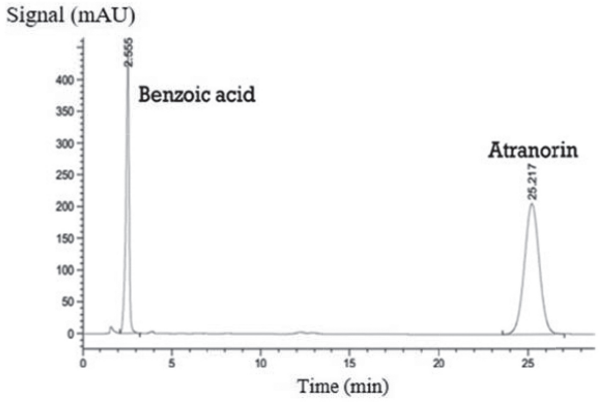

Fig. 1. Chromatogram of secondary metabolite in *Pyxine cocoes* in acetonitrile extraction. Identified peaks: benzoic acid (2.5 min) as internal standard and atranorin (25.2 min)

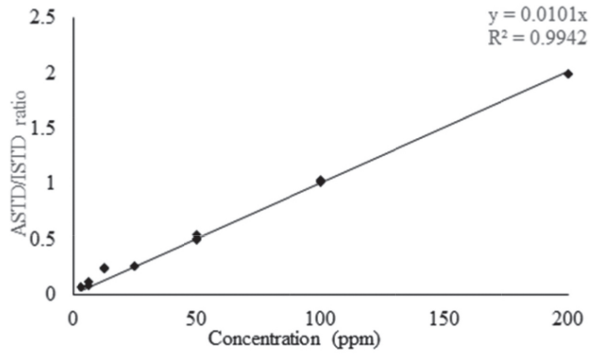

Fig. 2. Linearity curve of ASTD/ISTD ratio for HPLC determination of atranorin

The highest percentage change of average atranorin concentration between blank samples and other treatments was found in sample treated with sulfuric acid pH of 3.0, while the lowest percentage change was found in samples treated with sulfuric acid pH of 4.5 (Table 1). Similar to a study of Kovácik *et al*. (2011) who found that atranorin concentration in *H. physodes* was increased after being treated with sulfuric acid pH of 3.0. They suggested that lichens seem to respond to acid rain by synthesis specific metabolite such as atranorin. Bialonska and Dayan (2005) who found that atranorin concen-

Table 1. Average atranorin concentration after 15 days experiment: atranorin concentration in each treatment compared with blank samples, alphabet 'a' in superscript show non-significantly difference result ($p \leq 0.05$).

Treatment	Atranorin ± SD (ppm)	Percentage change (%)
Deionized water	125.57 ± 55.22[a]	12 %
pH 2.0	129.69 ± 45.63 [a]	16 %
pH 3.0	140.14 ± 21.90 [a]	25 %
pH 3.5	134.97 ± 22.72 [a]	21 %
pH 4.5	123.96 ± 14.61 [a]	11 %
blank sample	111.68 ± 26.61 [a]	

tration was decreased in higher polluted site but increased in lower polluted sites as detoxification reagent or precursor of other phenolic compounds. When compared with this study, atranorin concentration in a treatment pH of 2.5 was lower than a treatment pH of 3 and 3.5. Stronger acidic solution may affect lichen thallus, therefore its atranorin production was less than the other treatments. This re-

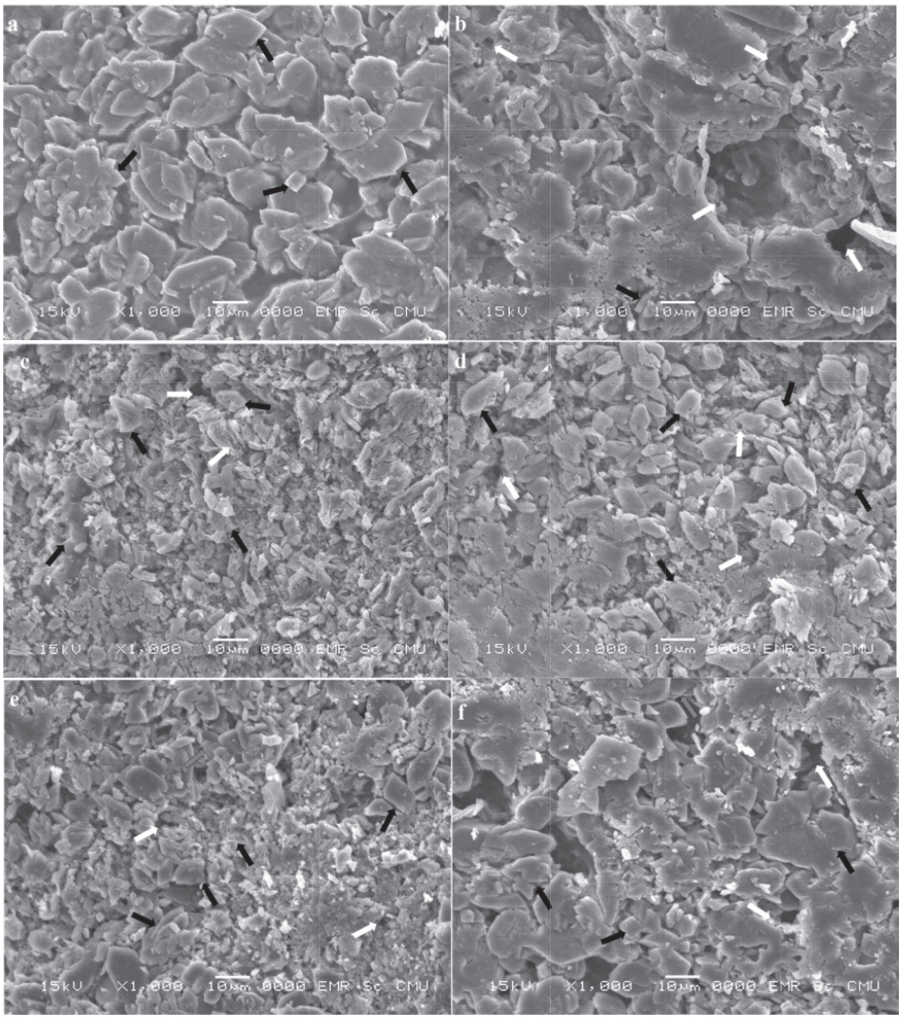

Fig. 3. SEM micrographs of upper surface of *Pyxine cocoes* in different treatment: a) DI water; b) sulfuric acid solution pH of 2.5; c) sulfuric acid solution pH of 3.0; d) sulfuric acid solution pH of 3.5; e) sulfuric acid solution pH of 4.5 and f) blank sample was not treated with anything. The white arrow shows the hole of upper surface or erosive zone or lack of crystal, the black arrow shows weddellite tetragonal shape and the light grey arrow shows whewellite monoclinic shape.

sult corresponded with Ohmura *et al.* (2009) who found lower atranorin concentration of *Parmotrema tinctorum* in polluted sites than in clean sites. They suggested that it might be related with the disintegration of the cortex and death of the photobionts.

However, Tukey's test in Table 1 shown non-significant difference between atranorin concentration in each treatment. The amount of atranorin concentration in DI water was higher than blank sample due to higher moisture in a treatment. Light and humidity are important factors to produced atranorin. High water potential and high light intensity activated the production of atranorin in fungal cortex (Armaleo *et al*., 2008).

Treated lichen samples were observed their structures under the SEM (Fig. 3). SEM micrographs show high density of calcium oxalate crystal with some weddellite tetragonal shape from all the samples. Crystal and upper surface structures in the blank sample and samples treated with DI water were defective, however it was not possible to clearly confirm that erosion of the crystals or upper surfaces were caused by acid deposition experiment or occurred before the experiment. However, sulfuric acid treatment in Fig 3 'b', 'c', 'd', and 'e' shows small size, crack, erosive and melted crystal that might be affected by an acid solution. Garty *et al.* (2002) found calcium oxalate crystals appear to disintegrate and provide a smaller crystal on the upper surface of thallus. Arocena *et al.* (2007) and Smieja-Król *et al.* (2014) found dissolution of calcium oxalate after treatment with acid. Consequently, when upper surface structure and calcium oxalate layer was damaged therefore acid solution can be absorbed into medullar layer faster and affect photobiont first. Hauck (2008) treated *Cetraria aculeata* and *Cetraria islandica* with SO_2 at pH 2.8 to 3.5 and water. It was found that chlorophyll fluorescence yield was highly reduced when treated with pH ≥ 3.3 in both species.

The unclear visible on the dissolution might cause of high density of calcium oxalate on the surface of *P. cocoes* which decreases the effect from sulfuric acid. That was showed with non-significant difference in atranorin concentration in each treatment. The covering of calcium oxalate on thallus may help lichen *P. cocoes* tolerate to acidic solution. Changing of lichen species may suggest in erosive effect of acidic solution in further study.

Acknowledgements

This study has been financial supported by Capacity Building Program for New Researcher 2018 from National Research Council of Thailand (NRCT), Environmental Science Research Center, Faculty of Science, Chiang Mai University and Graduate School, Chiang Mai University.

References

Arocena, J.M., Siddique, T., Thring, R.W. and Kapur, S. 2007. Investigation of lichens using molecular techniques and associated mineral accumulations on a basaltic flow in a Mediterranean environment. *Catena*. 70: 356–365.

Bialoñska, D. and Dayan, F.E. 2005. Chemistry of the lichen *Hypogymnia physodes* transplanted to an industrial region. *J. Chem Ecol*. 31: 2975–2991.

Burford, E.P., Hillier, S. and Gadd, G.M. 2006. Biomineralization of fungal hyphae with calcite ($CaCO_3$) and calcium oxalate mono- and dihydrate in carboniferous limestone microcosms. *Geomicrobiol J*. 23: 599-611.

Demirat, A.D., Yolcubal, I, Akyol, N. H. and Çobanoðlu, G. 2012. Biomonitoring of airborne metals using the lichen *Xanthoria parietina* in Kocaeli province, Turkey. *Ecol Indic*. 18: 632–643.

Dzubaj, A., Baèkor, M., Tomko, J., Peli, E. and Tuba, Z. 2008. Tolerance of the lichen *Xanthoria parietina* (L.) Th. Fr. to metal stress. *Ecotoxicol Environ Saf*. 70(2) : 319-26.

Farinha, M. M., Verburg, T. G., Freitas, M. C. and Wolterbeek, H. Th. 2009. Local and regional sources as given by aerosol measurements and biomonitoring at Sado estuary area, Portugal. *J Radioanal Nucl Chem*. 281(2): 299-305.

Garty, J., Kunin, P., Delarea, J. and Weiner, S. 2002. Calcium oxalate and sulphate-containing structures on the thallial surface of the lichen *Ramalina lacera*: response to polluted air and simulated acid rain. *Plant Cell Environ*. 25 : 1591-1604.

Hauck, M. 2008. Susceptibility to acidic precipitation contributes to the decline of the terricolous lichens *Cetraria aculeata* and *Cetraria islandica* in central Europe. *Environ Pollut*. 152: 731-735.

Hauck, M., Jürgens, S.R., Brinkmann, M. and Herminghaus, S. 2008. Surface hydrophobicity cause SO_2 tolerance in lichens. *Ann Bot*. 101: 531–539.

Jayalal, R.G.U., Ileperuma, O.A., Wolseley, P., Wijesundara, D.S.A. and Karunaratne, V. 2017. Correlation of atmospheric purity index to the diversity of lichens in the Horton Plains National Park, Sri Lanka. *Ceylon J Sci Biol Sci*. 46(2): 13-29.

Kosaniæ, M., Rankoviæ, B., Stanojkoviæ, T., Ranèiæ, A. and Manojloviæ, N. 2014. *Cladonia* lichens and their major metabolites as possible natural antioxidant, antimicrobial and anticancer agents. *Lebenson Wiss Technol*. 59 : 518-525.

Lackovièová, A., Guttová, A., Baèkor, M., Pišút, P. and Pišút, I. 2013. Response of *Evernia prunastri* to urban environmental conditions in Central Europe after the decrease of air pollution. *Lichenologist*. 45: 89–100.

Lohézic-Le Dévéhat, F., Legouin, B., Couteau, C., Boustie, J. and Coiffard, L. 2013. Lichenic extracts and metabolites as UV filters. *J Photochem Photobiol B.* 5(120): 17-28.

Ohmura, Y., Kawachi, M., Kasai, F., Sygiura, H., Ohtara, K., Kon, Y. and Hamada, N. 2009. Morphology and chemistry of *Parmotrema tinctorum* (Parmeliaceae, lichenized Ascomycota) transplanted into sites with different air pollution levels. *Bull Natl Sci Mus Ser B Bot*. 35(2) : 91-98.

Paoli, L., Guttová, A., Grassi, A., Lackovi, A., Sorbo, S., Basile, A. and Loppi, S. 2015. Ecophysiological and ultrastructural effects of dust pollution in lichens exposed around a cement plant (SW Slovakia). *Environ Sci Pollut Res Int*. 20 : 15891–15902.

Pinho, P., Augusto, S., Martins-Loução, M.A., Pereira, M.J., Soares, A., Máguas, C. and Branquinho, C. 2008. Causes of change in nitrophytic and oligotrophic lichen species in a Mediterranean climate: Impact of land cover and atmospheric pollutants. *Environ Pollut*. 154 : 380-389.

Purvis, O.W. 1984. The occurrence of copper oxalate in lichens growing on copper sulphide- bearing rocks in Scandinavia. *Lichenologist*. 16: 197–204.

Rajan, V.P., Gunasekaran, S., Ramanathan, S., Murugaiyah, V., Samsudin, M.W. and Din, L.B. 2016. Biological activities of four *Parmotrema* species of Malaysian origin and their chemical constituents. *J Appl Pharm Sci*. 6 : 036-043.

Saipunkaew, W., Wolseley, P.A., Chimonides, P.J. and Boonpragob, K. 2007. Epiphytic macrolichens as indicators of environmental alteration in northern Thailand. *Environ Pollut*. 146 : 366-374.

Smieja-Król, B., Janeczek, J. and Wiedermann, J. 2014. Pseudomorphs of barite and biogenic ZnS after phyto-crystals of calcium oxalate (whewellite) in the peat layer of a poor fen. *Environ Sci Pollut Res Int*. 21: 7227–7233.

Sransupphasirigul, N., Saipunkaew, W. and Chantara, S. 2012. Air quality mapping of Chiang Mai city using lichens as indicators. *1st ASEAN plus three graduate Research Congress*. 152–157.

Svoboda, D., Peksa, O. and Veselá, J. 2010. Epiphytic lichen diversity in central European oak forests: Assessment of the effects of natural environmental factors and human influences. *Environ Pollut*. 158 : 812–819.

The United States Environmental Protection Agency 2008. Acid deposition. EPA's report on the environment. National Center for Environmental Assessment, Washington, DC.

Zedda, L. 2009. Report on lichen sensitivity to air pollution with special reference to sulphur dioxide (SO_2). [online]. Available: http://www.docstoc.com/search/report on lichen sensitivity to air pollution. [6 March 2018].