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Air pollution affects lichen species richness, species density, relative growth form abundance and their secondary metabolite production: a case study in Kandy district, Sri Lanka

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Abstract Lichens are symbiotic associations between fungi and algae and/or cyanobacteria, consisting of three forms, *viz*: fruticose, foliose and crustose. Air pollution affects lichen diversity, percent cover, and density. The current study mainly compared the lichen species richness, density and relative abundance of each growth form among three selected sites with different degrees of pollution, *viz*: a site located in the Kandy City (S1), a site located 11 km away from the city (S2) and a site within a forest patch located ~12 km away from the Kandy city (S3). A random sampling method was used to collect lichens within a two km distance in the three selected sites. Percent cover and density were determined using a quadrat ladder. Acetone extracts of lichens were subjected to thin-layer chromatography (TLC), and secondary metabolites were identified by visualizing under UV (254 and 365 nm) and by Rf values. A total of 24 lichen species were collected (S1=8, S2=12, and S3=4). Percentage richness of crustose and foliose lichens was higher in S2 compared to S1, while *Leparia* sp. and *Lecanora* sp. were common in S1 and S2. Atranorin, salazinic acid, and zeorin were detected as common compounds from lichens in S1 and S2 sites, exhibiting photoprotecting and antioxidant properties. Fumarprotocetraric acid, which tolerates harsh environmental conditions, and Physodalic acid, which is produced in response to pollution stress were detected from lichens from S1. Norstictic acid was identified in lichens from S2. The results show a difference between the lichen community and secondary metabolites among the three sites.

Keywords: Air pollution, lichen density, lichen species richness, secondary metabolites, TLC analysis.

1 Introduction

Lichens are symbiotic associations between fungi (mycobiont) and algae and/or cyanobacteria (photobiont), which form a mutual biological union (Nash 2008). Lichens have different growth forms as crustose, foliose and fruticose (Nash 2008). Size of the lichen thallus/density, species diversity and the growth form composition

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(crustose, foliose, fruticose) are affected by many natural factors and man-made factors. Air pollution as a result of rapid urbanization has been found to affect the lichen diversity, percent cover, and density, and become one of the limiting factors for establishing successful lichen communities (Cislaghi and Nimis 1997, Nash 2008). Lichens are used as biomonitors and bio accumulators especially in Europe (Henderson-Sellers and Seaward 1979, Nimis and Purvis 2002). In addition to morphological and compositional changes that result from habitat changes, chemical profiles can also be affected.

Lichens are known to be sources of unique secondary metabolites. Secondary metabolites are produced by the fungal partner and secreted onto the hyphae as amorphous forms or crystals (Dharmadhikari and Chettiar 2010). Atranorin, parietin, usnic acid, fungal melanins accumulate in the cortex and physodic acid, physodalic acid, and protocetraric acid in the medullary layer. Secondary metabolites are produced as a result of limiting one or more nutrients and when growth slows down (Moore 1998). The production of secondary compounds may not serve specific functions, but they may have a selective benefit with multiple inadvertent ecological functions (Deduke *et al*. 2012). Lichen secondary metabolites are divided into many classes including amino acid derivatives, aliphatic acids, dibenzofurans, depsides, depsidones, depsones, sugar alcohols, macrolytic lactones, monocyclic aromatic compounds, chromones, terpenoids, steroids, carotenoids, diphenyl ethers, quinines, and xanthones (Huneck 1999). Over 800 secondary metabolites of lichens have been investigated which exhibit a wide variety of biological actions including antibiotic, antimicrobial, antiviral, anti-inflammatory, analgesic, antipyretic, anti-proliferative and cytotoxic effects (Boustie and Grube 2005, Basile *et al*. 2015).

Secondary metabolites are suggested to be mainly produced in response to extreme environmental conditions (Solhaug and Gauslaa 1996, Nybakken *et al*. 2004, McEvoy *et al*. 2006, Boustie *et al*. 2011). Sensitivity of lichen secondary metabolites to heavy metal accumulation has been observed which is suggested to play a general role in metal homeostasis and pollution tolerance. Białonska and Dayan (2005) reported a decrease in the levels of atranorin, physodic acid and hydroxyphysodic acid in thalli of *Hypogymnia physodes* transplanted to the vicinity of a chemical plant producing chromium, phosphorous and sulfur compounds. However, a significant increase in the level of physodalic acid was observed suggesting that this compound is produced in response to the pollution stress. In *Evernia mesomorpha* and *Ramalina menziesii*, usnic acid and divaricatic acid significantly increased the intracellular uptake of Cu^{2+} but reduced the intracellular uptake of Mn^{2+} (Hauck *et al*. 2009). These compounds were suggested to facilitate the survival of those two particular lichen species. Pawlik-Skowronska and Backor (2011) investigated the effect of Zn/Pb-pollution on secondary metabolites production in *Hypocenomyce scalaris*, *Cladonia furcata* and *Lepraria* spp. and suggested that Zn and Pb ions tolerate lichen species that contain higher amounts of lecanoric, fumarprotocetraric, stictic, constictic acids, and atranorin. Nakajima *et al*. (2015) reported that the relative concentrations of stictic and norstictic acids in lichen samples collected at Cu-

polluted sites were significantly lower compared to those at control sites. The coordinated production of usnic acid with salazinic acid was investigated by Valencia-Islas *et al*. 2007 and Amo de Paz *et al*. 2010 and show that usnic acid and salazinic acid share similar effects due to air pollution and antioxidant behaviour.

However, studies to elucidate the impact of habitat disturbances on lichen metabolite production coupled with lichen diversity are intermittent globally and are rare in the Sri Lankan context, though about 700 lichen species have been recorded in Sri Lanka (Weerakoon 2015). Therefore, the goal of the current study was to investigate the effect of environmental changes resulting from anthropogenic disturbances on lichen diversity and its biochemistry. The objectives of the current study were to compare lichen species richness and density and to assess the number and type of secondary metabolites produced by each species collected from three sites comprising of different levels of air pollution.

2 Material and Methods

2.1 Selection of study sites

Kandy city has been declared as one of the most polluted cities in Sri Lanka with NO_2, SO_2, O_3 concentrations exceeding the permitted Sri Lankan standard levels for ambient air quality and subjected to transboundary pollution in certain months of the year (Abeyratne and Illaperuma 2006). Elangasinghe and Shanthini (2008) reported a positive correlation between atmospheric particular matter less than 10 μm in size (PM10) concentration in Kandy in relation to traffic intensity.

Site 1 (S1) was around the Kandy Lake, which is situated in the middle of the city of Kandy (7°17'25.4"N 80°38'23.7"E) where heavy traffic is observed throughout the day and night, and with a recorded value of 200 $\mu g/m^3$ of atmospheric particular matter less than 10 μm in size (PM10) (Elangasinghe and Shanthini 2008). University of Peradeniya, which is located on the opposite basin of the Hantana mountain range and at the edge of Upper and Lower Hantana Forest reserves was selected as the second site (S2) (7°15'17.6"N 80°35'55.5"E). Since the traffic flow is less along the road that runs across the University, the atmospheric particular matter less than 10 μm in size (PM10) was recorded as 25 $\mu g/m^3$ (Elangasinghe and Shanthini 2008). Gannoruwa forest reserve, which is spread over the mountain facing the Upper and Lower Hantana forest reserve was selected as the third site (S3) (7°17'16.0"N 80°35'48.0"E). It is isolated and located about 5 km away from the less busy highway. Based on the location of the three sites, disturbances to the lichen communities inhabiting these sites were assumed to be varied, especially with respect to air quality (Figure 1).

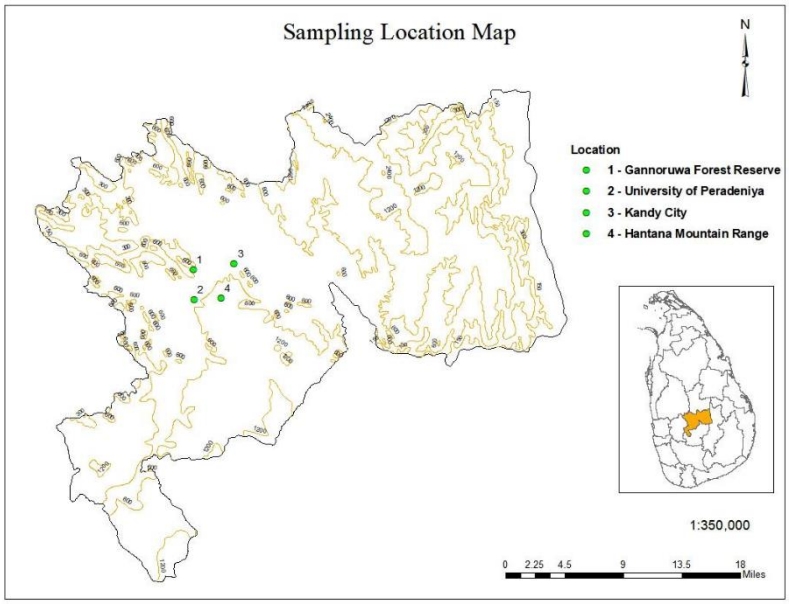

Fig 1. Three sampling sites (S1, S2, S3) and location of Hantana mountain range in the Kandy district, Sri Lanka

2.2 Comparison of relative abundance of growth form and lichen species richness

Corticolous lichen species richness in the three selected sites was determined using a quadrat ladder (each having 10×10 cm^2 contiguous quadrats). Substrate tree species was recorded, and the quadrat ladder was placed on the tree trunk 1.5 m above the ground level. Four aspects *viz*: N, E, S, W of the trunk were examined and the presence of lichens according to their thallus forms; crustose, foliose and fruticose within the quadrat was recorded. Lichen richness was determined by recording lichen species on 10 randomly selected tree species as the above-mentioned procedure and the relative abundance of each thallus forms were calculated using the following formulae.

$$\text{Relative abundacne of Growth Form} = \frac{\text{No. of lichen of a paricular growth form}}{\text{Total no. of lichen belonging to all growth forms}} \text{ X } 100$$

Percent coverage of each lichen species and lichen forms were visually estimated by placing the quadrat ladder using the method described above. Density of each lichen species and growth forms within the quadrat ladder was obtained using the following formula.

$$\text{Density} = \frac{\text{No. of total individuals of a particular lichen species/form}}{\text{Size of the quadrat}}$$

2.3 Comparison of chemical profiles of lichen species

Sample collection

Three forms of corticolous lichen samples were collected from the selected sites by using random sampling method. Site1 was a "~2 km stretch along the road running around the Kandy Lake in the City of Kandy (S1) (Figure 1). Site 2 was at the University of Peradeniya along a ~2 km stretch of the road running through the University (S2), and Site-3 was Gannoruwa forest reserve along the foot path ~2 km (S3). Samples were collected from ten trees from each site and four 10 x10 quadrats per tree. Therefore, the total number of quadrats is 40 per site. For each lichen sample, three replicate samples were collected for identification and chemical analyses. Subsequently, the samples were sorted out according to thallus form, air dried and stored at room temperature until processing. Samples were identified with standard taxonomic keys using morphological and chemical characteristics (Weerakoon 2015).

Thin Layer Chromatography (TLC)

The extract was prepared under sterile conditions according to the method described by Culberson (1972). Secondary metabolites in all samples were extracted using acetone and identified according to spot characteristics as follows (Orange *et al*. 2001). One millimeter of each acetone extract was spotted on TLC plates with a capillary tube. TLC plates were allowed to dry for 30 minutes to one hour. Each plate was run in the developing chamber with the solvent system A; toluene/ethyl acetate/formic acid (139:83:8 v/v/v). TLC plates were observed under a UV-illuminator (254 nm and 365 nm) and spot characteristics were recorded. Sulfuric acid (10%) was sprayed on TLC plates and plates were oven-dried at 80^{o}C for 10 minutes to develop the characteristics further. Secondary metabolites were determined by comparison with known characteristics (Orange *et al*. 2001), by using a standard for Rf comparison. A mixture of acetone extract of usnic acid (Rf 6) from *Usnea* sp. and Norstictic acid (Rf 4) from *Ramalina* sp. was used as the standard reference (Supplementary file 1).

3 Results

3.1 Comparison of relative abundance of growth forms and lichen species abundance

Based on a few previous studies on lichen diversity and air pollution (Cislaghi and Nimis 1997, Nash 2008), higher species richness of lichens and an abundance of fruticose lichens were expected in the Gannoruwa forest reserve (S3), while species richness and fruticose and foliose lichen abundance were expected to be lowest

around the Kandy Lake (S1). Expected patterns were observed in polluted (S1) and semi polluted (S2) habitats in terms of lichen richness and density. As shown in Table 1 and 2, the total number of species was higher in the S2 site and the least number of species was found in S3. Crustose lichens were found in all sites and foliose lichens were found mainly in S2 and few in S1. Fruticose lichens were absent in all sites. Relative abundance of crustose and foliose lichens was higher in S2 compared to S1.

Table 1: Percentage diversity and distribution of different forms of lichen thalli in the three Sites

Site	Total number of species	Thallus form (Relative abundance %)		
		Crustose	Foliose	Fruticose
(S1)	8	37.5	62.5	ab
(S2)	12	41.66	58.33	ab
(S3)	4	100	ab	ab

ab = Absent

Table 2: List of lichen species identified from each site, their growth form, and identified secondary metabolites in each lichen species using thin-layer chromatography.

Site	Growth form	Species	Secondary metabolites
S1	Crustose	*Graphis* sp. 2	Physodic acid, Fumarprotocetraric acid, Acetylportentol
	Crustose	*Lecanora* sp.	Physodic, unidentified compound
	Crustose	*Lepraria* sp.	Physodalic, Zeorin
	Foliose	*Leptogium* sp.	No compound
	Foliose	*Lobothallia* sp.	Norstic acid, Zeorin
	Foliose	*Parmotrema* sp.	Atranorin, Salazinic, Zeorin
	Foliose	*Physcia* sp.	Atranorin, Zeorin
	Foliose	*Pyxine* sp.	Lichexanthone
S2	Crustose	*Cryptothecia* sp.	Chiodectonic acid
	Crustose	*Graphis* sp. 2	Leanoric acid, Stictic acid, Norstictic acid
	Crustose	*Lecanora* sp.	Constictic acid, Salazinic acid, Stictic acid
	Crustose	*Lepraria* sp.	Atranorin, Constictic acid, Stictic acid, Zeorin
	Foliose	*Heterodermia* sp.	Zeorin
	Foliose	*Leptogium* sp.	No compound
	Foliose	*Parmotrema* sp. 1	Salazinic acid
	Foliose	*Parmotrema* sp. 2	Atranorin, Salazinic, Zeorin
	Foliose	*Parmotrema* sp. 3	Atranorin, Lecanoric acid
	Foliose	*Physcia* sp. 1	Salazinic acid, Zeorin
	Foliose	*Physcia* sp. 2	Atranorin, Zeorin
S3	Crustose	*Megalospora* sp. *Porina* sp. 1 *Porina* sp. 2 *Trypethelium* sp.	Atranorin

Note: S1: ~2 km stretch along the road running around the Kandy Lake in the City of Kandy; S2: University of Peradeniya along a ~2 km stretch of the road running through the University; S3: Gannoruwa forest reserve along the foot path ~2 km.

3.2 Percentage cover and average density of lichen

Comparison of average percent cover and average density of lichens found in the three sites are shown in Figure 2. The highest percent cover (63%) and the least average density (13.42) values were observed in S3. The reason for this observation is the large healthy patches of crustose lichens covering the tree trunks. Moderate percent cover (40.12%) and moderate average density (22.35) values were observed in S2 and the least percent cover (7.07%) and the highest average density (28.47) was observed in S1.

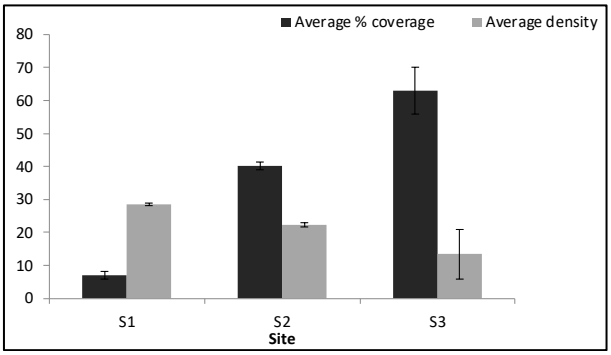

Fig 2. Average percent cover and average density of lichens collected from the three sites.
S1: Kandy city, S2: University of Peradeniya, S3: Gannoruwa forest reserve.

3.3 Composition of secondary metabolites in lichen species collected from the three sites

Lichen species in S2 and S1, showed higher number of secondary metabolites than lichens found in S3 (Table 2). Only atranorin was detected from the crustose lichen species (*Megalospora* sp., *Porina* sp. 1, *Porina* sp. 2 and *Trypethelium* sp.) from the S3. Atranorin, salazinic acid, and zeorin were detected as common compounds to S2 and S1, which has photoprotecting and antioxidant properties. In S2, norstictic acid was identified, which is produced under low light levels. In S1, fumarprotocetraric acid (suggested as to tolerate harsh environmental conditions) and physodalic acid (suggested as produced in response to pollution stress) were identified (Table 2). Lichen species *Leparia sp.* and *Lecanora sp.* were observed in both S2 and S1 sites, but their secondary metabolites profiles were different as shown in Table 2.

4 Discussion

The current study compared the species richness, density, relative abundance of different growth forms and secondary metabolite profiles of the lichen species collected from three different sites where air pollution levels are recorded and

assumed to be different based on previous records. The selection of the polluted site was based on the study carried out by Abeyratne and Illaperuma (2006) and Elangasinghe and Shanthini (2008).

A higher number (density) of crustose lichen species was observed in polluted habitat (S1) compared to the semi-polluted habitat (S2) even though it exhibited a fewer number of crustose lichen species. This observation supports the previously established fact that crustose lichens tolerate air pollution (Pyatt 1970, Nash 1974). This also confirms that the air pollution levels are high around the Kandy Lake (S1) in comparison to the University (S2). Absence of fruticose lichen species in both habitats also suggests that both habitats are disturbed and polluted. However, the current study observed only four crustose lichens species in the sample site in the Gannoruwa forest reserve (S3), and the least number of species were collected from this site during this study in comparison to the other two sites. Possible reasons for this observation could be the less amount of sunlight due to the canopy cover and higher atmospheric moisture content creating unique environmental conditions suitable for a limited number of lichen species exerting a competition so that other species cannot survive on the tree barks rather than air pollution. The micro-environmental conditions prevailing in the forest area may augment the successful spread of these four species. Therefore, in future studies, it is recommended that the sampling area should be increased, expanding to the other side of the Gannoruwa forest. In addition, the nearby natural forest patches in Kandy district including Udawaththa forest reserve and Hanthana sub mountain forest should also be investigated, to see whether similar patterns are observed. Even though only four crustose species were present in Gannoruwa forest (S3) their percent cover was very high since the tree barks were covered with large patches of healthy lichen thalli. This further indicates that these four crustose species are well established and dominate the habitat. Percent cover of the crustose and foliose lichens in the semi polluted area (S2) was higher than that in the polluted area (S1), indicating that extreme environments can affect the thallus size, hence the growth rate of lichens.

Based on the fact that secondary metabolites are induced under stress conditions (Solhaug and Gauslaa 1996, Boustie *et al*. 2011, Nybakken *et al*. 2004, McEvoy *et al*. 2006), our study expected an increase in the number of secondary metabolites produced by lichens under polluted environments. Similarly, crustose lichens were expected to produce more compounds than foliose since crustose tolerate harsh conditions (Pyatt 1970, Nash 1974). Production of secondary metabolites could be one possible mechanism where crustose lichens withstand harsh environments. But both in semi polluted (S2) and polluted habitats (S1), crustose and foliose lichen species showed a similar number (three) of secondary metabolites. From the crustose lichens collected from the unpolluted area (S3), atranorin was identified as the only secondary metabolite that exhibits photoprotecting and antioxidant properties. A previous study has recorded the amount of atranorin in the cortex of *Parmotreama hypotropum* positively correlating with the amount of yearly light reaching the thallus (Armaleo *et al*. 2008). Even though there is low light accessing the tree trunk due to

high canopy cover in the Gannoruwa forest area (S3), they still produce atranorin to give protection to the light-sensitive algal partner, however, the quantity may be minimal. This might be an indication that the forest is providing favourable conditions. Atranorin was detected in a few lichen samples in all three habitats, and extended studies into investigating the concentration of secondary metabolites would give an apparent effect of air pollution on secondary metabolites production. Only two lichen species (crustose) were found to be common in both semi polluted (S2) and polluted (S1) areas. These showed a difference in the numbers of secondary metabolites, and their composition (*Leparia* sp. and *Lecanora* sp.). Physodalic acid was identified as one secondary metabolite in *Leparia* sp., and physodic acid was identified in *Lecanora* sp., which was collected from the polluted area (S1). Previous studies reported that the level of physodic acid decreased while physodalic acid increased in response to heavy metal pollution (Białonska and Dayan 2005).

Under polluted conditions, both species produce physodalic acid which suggests an increase in the levels in response to pollution stress. Physodic acid is shown to decrease in the levels under chemical pollution (chromium, phosphorous, and sulfur compounds) (Białonska and Dayan 2005), which was observed in the current study. This indicates that the same species may produce different metabolites under different pollution levels. This suggests that the secondary metabolite composition can be affected by different pollution levels, and lichens growing under low air quality can be a source of unique metabolites that have significant applications. Therefore, further detailed investigation (identification and quantification) on physodalic acid production in lichen species is also important within this context.

Apart from the number and the type of metabolites, concentration differences of the same compound can be triggered by environmental differences, which was not determined in the current study. One of the best methods to identify exact compounds and quantify them is High-Performance Liquid Chromatography (HPLC). According to Pyatt (1970), the crustose and *Leprosa* lichens are more tolerant regarding air pollution than foliose and fruticose forms. The current study provides the same evidence by the presence of *Leparia* sp. in both semi polluted (S2) and polluted (S1) habitats. Bajpai *et al*. (2010) suggested *Leparia* sp. which accumulate a high concentration of almost all the heavy metals can be used to determine the heavy metal pollution in the dry and warm climate of Central India that has similar climatic conditions as Sri Lanka. Therefore, *Leparia* sp. may be of value to be used as an indicator species for future studies to determine the ambient air quality in a particular area in Sri Lanka.

Atranorin, salazinic acid, and zeorin were common compounds detected in lichens collected from semi polluted (S2) and polluted habitat (S1), which has photoprotecting and antioxidant properties. Previous studies (Deduka *et al*. 2012, Shukla *et al*. 2016) reported higher abundance and frequency of *Everniastrum cirrhattum* with increasing altitude and detected the presence of a higher quantity of photoprotecting and antioxidant chemicals, especially salazinic acid. This explains the harsh environmental conditions of both semi polluted (S1) and polluted (S2) sites.

Along the Galaha road, the University of Peradeniya (S2) mature trees were observed with a large canopy cover compared to trees present around the Kandy Lake (S1); hence lower level of light is received to the lichen thallus in semi polluted site (S2) when compared to the polluted site (S1). Therefore, the concentration of these photoprotecting compounds (atranorin, salazinic acid, zeorin) are expected to be higher in the polluted site. Armaleo *et al*. (2008) detected norstictic acid on the medullary hyphae and found a negative correlation with yearly light levels. The authors interpreted that the higher quantities of the medullary compound under lower light levels may be an adaptative link with the need to produce these hydrophobic compounds when water potential increases within the lichen thallus (from low light levels) to facilitate the efficient carbon dioxide diffusion to the algae. As light levels decrease, the water potential in the lichen thallus increases, and at the same time, the requirement of hydrophobic compounds also increases. In semi polluted site (S2), *Graphis* sp.1 were identified with norstictic acid and thus provided little evidence of its advantage under low light levels. Fumarprotocetraric acid that is suggested to be produced to tolerate harsh environmental conditions (Culberson *et al*. 1977) was identified only in *Graphis* sp. 2, which was collected from the polluted site (S1).

As future directions, the concentration of SO_2, NO_2, and O_3 should be determined in the three sites to confirm the pollution level. In addition to the Gannoruwa forest reserve, other forests such as Udawaththa forest and upper Hanthana forests need to be examined to find out whether the same trend of lichen diversity is present there. The current study supports the primarily established fact that lichen species diversity and growth form composition decrease with increasing environmental stress, in this case, air pollution. However, studies on the relationship between air pollution levels, lichen metabolite production, including lichen species richness are not prevalent both globally and in the Sri Lankan context. Based on the available literature, this is the third study that has been carried out to determine the effect of pollution stress on secondary metabolites of lichens globally and the first of such studies in Sri Lanka. This is one of the few studies to investigate the possible impact of low air quality on lichen community in the Kandy city, which has been declared as the second most polluted city in Sri Lanka by the Central Environmental Authority, Sri Lanka.

One of the major limitations of this study was measuring the levels of gaseous air pollutants in each selected site. However, due to the unavailability of resources to monitor the air quality, we went for an alternative method to get already recorded data from possible places. In addition, after careful observation of the location of three sites with respect to traffic conditions, we made an educated guess that the selected sites must have three distinctive levels of air pollution. After an intensive search, we found some published data to support our guess regarding particulate matter (Elangasinghe and Shanthini 2008) in two of the areas in our study.

Furthermore, a follow-up study conducted by our research group detected a higher number of heavy metal types on lichen thalli collected from around Kandy Lake than those collected from the same study area in the University of Peradeniya (ESM

Edirisinghe 2020, Unpublished data). Based on these additional data, we are very confident that the air pollution levels in the three selected sites are significantly different from each other and vary as we assumed. Further, using the Atmospheric Purity Index could have been ideal in this situation. However, the study design and the data collected during the study do not support calculating API.

The current study is an initiative to investigate the relationship between air pollution and lichen metabolite production will be a platform for extensive research on identifying and quantifying unique secondary metabolites produced under certain environmental conditions and further studies will be helpful to identify the mechanism behind the survival of lichens under stress conditions.

5 Conclusions

This study mainly compared the lichen species richness, density, and relative abundance of each growth form among three selected sites with different degrees of pollution levels. A higher number (density) of crustose lichen species was observed in polluted habitat (S1) compared to the semi-polluted habitat (S2), even though it exhibits a fewer number of crustose lichen species. Not finding fruticose lichen species in both habitats suggests that both habitats are disturbed and polluted. Percentage richness of crustose and foliose lichens was higher in semi-polluted habitat (S2) compared to polluted habitat (S1). *Leparia* sp. and *Lecanora* sp. were common to S1 and S2, but their secondary metabolites profile differed in each site. The results show a difference between the lichen community and secondary metabolites among the three sites. The current study is an initiative to investigate the relationship between air pollution and lichen metabolite production will be a platform for extensive research on identifying and quantifying unique secondary metabolites produced under certain environmental conditions and further will be helpful to identify the mechanism behind the survival of lichens under stress conditions.

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