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Proximate and elemental analysis of *Ramalina conduplicans* Vain. (Ramalinaceae) and *Parmotrema tinctorum* (Nyl.) Hale (Parmeliaceae)

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ABSTRACT

The objective of the present study was to determine proximate and mineral composition of two macrolichens namely Ramalina conduplicans Vain. (Ramalinaceae) and Parmotrema tinctorum (Nyl.) Hale (Parmeliaceae). The powdered lichen materials were subjected for proximate analysis to estimate moisture, ash, total protein, total carbohydrate, crude fibre and crude fat content. The content of mineral elements was estimated by ICP-OES after microwave digestion. The content of moisture, ash, crude fibre and protein was higher in P. tinctorum whereas crude fat and carbohydrate content was higher in R. conduplicans. The content of all elements except calcium and copper was higher in P. tinctorum. The content of calcium and potassium was highest in R. conduplicans and P. tinctorum respectively among major elements. In case of minor elements, iron and nickel were found to be highest and least respectively in both the lichens. In conclusion, the results of proximate and mineral content of lichens of this study indicate that the utilization of these lichens in food can be nutritionally advantageous.

Key words: Macrolichens, *Ramalina conduplicans*, *Parmotrema tinctorum*, Proximate composition, Elemental analysis, ICP-OES

INTRODUCTION

Lichens represent a symbiotic interaction between a fungal partner (a mycobiont) and a photosynthetic partner (a phytobiont represented by algae or cyanobacteria). More than 20000 species of lichens are known. The lichens are known to inhabit diverse ecosystems ranging from arctic tundra to desert climates. They are ubiquitous and are found on barks, stems, leaves, in soil and water but often grow in conditions less favorable for other organisms. Throughout history, these lichens have been used as food, folk medicine, dyes, in the production of alcohol and perfume industry. Lichens are used for a number of years as natural bioindicators for various heavy metals and as sources of information for environmental monitoring. Lichens produce a number of characteristic secondary metabolites called lichen substances exceeding >1000 in number. These substances are produced mostly by fungal partner and are unique to lichens and not found in other organisms. The extracts and metabolites of lichens are known to display a range of biological activities such as antimicrobial, antioxidant, anti-inflammatory, analgesic, antiherbivore, antitumor, allelopathic, enzyme inhibitory, antipyretic etc **[1-4]**.

The utilization of lichens as principal food by humans is limited. Often, their use is restricted to conditions like famine in which survival becomes a critical situation. Redzic *et al*. **[5]** reported the utilization of 7 lichen species including *Evernia prunastri* and *Usnea* sp. by people in Bosnia during war conditions. Globally, several species of lichens are used as food or to flavor foods. Lichens such as *Parmelia*, *Heterodermia*, *Ramalina*, *Rimelia*, *Parmotrema*, *Usnea*, *Everniastrum* and others are used to prepare several Indian dishes such as biryani, curries etc. **[6-9]**. In Yunnan province of southwestern China, *Ramalina conduplicans* is used to prepare a traditional cold dish served at marriage banquets and in a stir-fried pork dish **[10]**. The Rai and Limbu communities of East Nepal use *R.*

conduplicans traditionally for preparation of food **[11]**. In many places of India, the lichen is used as a spice **[6].** Ethnic minorities in Naban River Watershed National Nature Reserve, Yunnan, China consume a species of *Ramalina* as wild food plant **[12]**. Lichens belonging to 5 genera are used as edible wild plants by Tibetans in Shangri-la region, Yunnan, China **[13]**. Besides use in human nutrition, lichens are also preferred by animals. In winter, lichens such as species of *Cladonia*, *Cetraria* etc., form the major source of winter pastures for grazing animals such as caribou and rain deer **[14,15]**. Keeping in mind the potential uses of lichens as food and in the preparation of food, the present study was focused on determining proximate and mineral element composition of two macrolichens *viz*., *Ramalina conduplicans* Vain. (Ramalinaceae) and *Parmotrema tinctorum* (Nyl.) Hale (Parmeliaceae).

EXPERIMENTAL SECTION

Collection and identification of lichens

The lichens *R. conduplicans* and *P. tinctorum* were collected during December 2013 from Hosalli and Maragalale of Shivamogga district respectively. The lichens were identified on the basis of morphological, anatomical and chemical tests. Color reactions were performed on the cortex and medulla by using 10% potassium hydroxide (K), Steiner's stable paraphenylenediamine solution (P) and calcium hypochlorite solution (C). Thin layer chromatography (TLC) was performed to identify characteristic secondary metabolites using solvent system A (Benzene:1,4-Dioxane:Acetic acid in the ratio 90:25:4). The spots were marked, Rf values were calculated and the compounds were identified **[16-18]**.

Proximate composition of lichens

Moisture content

A known quantity (10g) of powdered lichen material was taken in a pre-weighed flat-bottom dish (W1) and kept overnight in hot air oven at 100°C, cooled and weighed (W2). The moisture content of lichens was calculated using the formula:

Moisture content = ([W2-W1] / weight of material)x100 **[19]**.

Total ash

10g of lichen material was placed in a pre-weighed silica crucible (W1). The crucible was heated over a low flame (till the lichen material was completely charred) followed by heating at 600°C in a muffle furnace for about 3–5 hours. The crucible was cooled in a desiccator and weighed (W2). In order to ensure complete ashing, the crucible was heated again in the furnace for half an hour, cooled and weighed. This was repeated consequently till the weight of ash become constant (ash became white or grayish white). Total ash content was calculated using the formula:

Total ash content = (weight of ash [W2-W1] / weight of material)x100 **[19]**.

Crude fibre

Crude fibre estimation is based on treating the moisture and fat-free sample with dilute acid (1.25%) and then with dilute alkali (1.25%) which is similar to the gastric and intestinal action in the process of digestion. In this method, 2g of moisture and fat-free lichen material was added to 200 ml of 1.25% H_2SO_4 and boiled for 30 minutes. The content was filtered through linen cloth and the residue was washed with boiling water till no acidic. The residue was further treated with 200ml of 1.25% NaOH and boiled for 30 minutes. The content was filtered through linen cloth, washed with boiling water and then with 1% HNO_3 and again with hot water. The residue obtained was taken in a pre-weighed silica crucible (W1), heated at 600^oC until complete ashing and the crucible was again weighed (W2). Crude fibre content was calculated using the formula:

Crude fibre content = (weight of residue [W2 -W1] / weight of material)x100 **[19]**.

Crude fat

2g of moisture free lichen material was extracted using petroleum ether (boiling point 40-60^oC) in a Soxhlet extractor for 24 hours (or until a drop taken from the drippings left no greasy stain on the filter paper). Later, petroleum ether was drained into another pre-weighed (W1) container. The container was kept in hot air oven until it was free of petroleum ether, cooled in desiccator and weighed (W2). The difference in the weight was taken as fat content. The crude fat (%) in the lichen materials was calculated using the formula:

Crude fat (%) = (Weight of fat [W2-W1] / Weight of sample taken)×100**[19]**.

Protein content

The micro Kjeldahl method was performed to estimate the crude protein content of lichen materials **[19,20]**. Here, 2g of oven-dried lichen material in a Kjeldahl flask was added with 30ml of concentrated H_2SO_4, 10g potassium sulphate and 1g copper sulphate. The mixture was heated gently first and then strongly once the frothing had ceased. Further, the colourless or clear solution was heated for another hour, cooled and diluted with distilled water to the mark in a 100ml volumetric flask. 10ml of aliquot of the digest was measured into the decomposition chamber of the distillation apparatus and 15ml of 40% NaOH was added and the ammonia released was trapped into 20ml of 2 % boric acid solution containing mixed indicator. A colour change from pink to green was observed as the ammonia being trapped. Distillation was continued for 5 minutes and the boric acid-ammonia solution so obtained was titrated against 0.1N HCl. The % of nitrogen was calculated by using the formula:

% Nitrogen = (T–B) x N x 0.014 x D x 100 / Weight of the sample x V, where T is sample titration reading, B is blank titration reading, N is normality of HCl, D is dilution of sample after digestion, V is volume of sample taken for distillation and 0.014 is milliequivalent weight of nitrogen.

% crude protein was calculated using the formula:

% crude protein = 6.25* x % nitrogen, where * is correction factor.

Total carbohydrate content

Percentage carbohydrate was calculated by using the formula:

Carbohydrate (%) = 100 – (percentage of ash + percentage of moisture + percentage of fat + percentage of protein) **[19]**.

Nutritive value

Nutritive value of lichens was determined by using the formula:

Nutritive value = (4 x percentage of protein) + (9 x percentage of fat) + (4 x percentage of carbohydrate) **[19]**.

Mineral composition of lichens

1g of powdered lichen material was digested using 10ml of ultrapure metal free nitric acid in a microwave digester (CEM). After digestion, the content was filtered through Whatman filter paper No. 1 and diluted to 25 ml with distilled water. The digested lichen sample was aspirated into ICP-OES (Agilent Technologies 700 series, US) to estimate major elements *viz*., Calcium (Ca), Potassium (K), Sodium (Na), Phosphorus (P) and Magnesium (Mg) and minor elements *viz*., Manganese (Mn), Iron (Fe), Zinc (Zn), Nickel (Ni), Chromium (Cr) and Copper (Cu). The standards were prepared by diluting stock multi-elemental standard solution in nitric acid **[21]**. Instrument configuration and experimental conditions are presented in Table 1.

Table 1. ICP-OES operation conditions

Power (kW)	1.2
Plasma flow (L/min)	15.0
Auxiliary flow (L/min)	1.50
Nebulizer flow (L/min)	0.75
Sample flow rate (L/min)	1.5
Replicate read time (s)	3.00
Instrument stabilization delay (s)	15.0
Sample uptake delay (s)	10.0
Pump rate (rpm)	15.0
Rinse time (s)	10.0
Spray chamber	Cyclonic type
Elements, wavelengths (nm)	Ca (422.673), Cu (327.395), Na (589.592) Cr (267.716), Fe (238.204), K (766.491), Mg(279.553), Mn (257.610), Ni (231.604), Zn (213.857), P (213.618)

RESULTS AND DISCUSSION

Characteristics of lichens used in this study

The morphological, anatomical and chemical characteristics of selected lichens are presented in Table 2.

Table 2. Description of *R. conduplicans* and *P. tinctorum*

Characteristics	*R. conduplicans*	*P. tinctorum*
Form	Fruticose	Foliose
Habitat	Corticolous (bark of areca tree)	Corticolous (bark of cashew tree)
Thallus description	Thallus 3-5cm long, pendulous, flattened, greenish grey, branched; upper side smooth, scarcely pseudocyphellate; lower side rugose, with raised, round, prominent pseudocyphellae; soredia and isidia absent, chondroid tissue present and uneven in thickness, distinctly cracked into hyphal bundles; medulla solid, white; pith filled	Large loosely adnate, membranous, broad, lobes irregular, rotund; margins crenate, eciliate; upper surface grey, smooth, isidiate; lower surface minutely wrinkled, rough, black, erhizinate; rhizines sparse, coarse at the centre
Color tests	Cortex K-; Medulla K-, C-, KC -, Pd+ yellow	Cortex K+ yellow; Medulla K-, C +red, KC +red, Pd -
TLC	Usnic acid, Salazinic acid, Sekikaic acid	Lecanoric acid, Atranorin

Proximate composition of lichens

The proximate composition of powdered lichen materials is shown in Table 3. Moisture, ash, crude fibre and protein content of *P. tinctorum* were higher than that of *R. conduplicans*. The content of crude fat and carbohydrate was higher in *R. conduplicans* when compared to *P. tinctorum*. The nutritive value was high in *R. conduplicans*.

Table 3. Proximate composition of *R. conduplicans* and *P. tinctorum*

Parameter	*R. conduplicans*	*P. tinctorum*
Moisture (%)	8.86	9.12
Ash (%)	4.01	6.15
Crude fibre (%)	5.86	16.36
Crude fat (%)	2.1	1.3
Protein (%)	5.95	11.3
Carbohydrates (%)	79.08	72.13
Nutritive value (Cal/100g)	359.02	345.42

Mineral content of lichens

Table 4 shows the content of major and minor mineral elements of lichens selected. Calcium and copper were detected in high quantity in *R. conduplicans* whereas other elements were found in high quantity in *P. tinctorum*. In case of *R. conduplicans*, calcium and iron were detected in high quantity among major and minor elements respectively. However, in case of *P. tinctorum*, potassium and iron were in high quantity among major and minor elements respectively.

Table 4. Mineral content (in ppm) of *R. conduplicans* and *P. tinctorum*

Minerals	*R. conduplicans*	*P. tinctorum*
Calcium	7040.20	2334.13
Potassium	2548.92	2931.82
Sodium	154.31	370.58
Phosphorus	737.22	852.08
Magnesium	561.98	884.34
Iron	1009.64	8250.52
Copper	30.33	30.15
Zinc	46.11	55.81
Manganese	32.23	233.75
Nickel	1.92	9.85
Chromium	6.14	21.60

Moisture content is an important parameter which is important as far as the shelf-life of any food is concerned. Foods with more moisture content undergo spoilage very easily than foods with low moisture content as they are more prone to microbial spoilage **[22]**. In this study, the moisture content of both the lichens is more or less similar (8.86% and 9.12% for *R. conduplicans* and *P. tinctorum* respectively). The moisture content of both lichens was shown to be lesser when compared to moisture content of *R. conduplicans* (16.4%), *R. hossei* (16%) and *P. pseudotinctorum* (15.2%) of earlier studies **[23-25]**. Comparatively the ash content was higher in *P. tinctorum* (6.15%) than *R. conduplicans* (4.01%). The ash content represents the mineral composition of samples **[22]**. The ash content of both lichens was found lesser than that of ash content of *R. conduplicans* (10%), *R. hossei* (12.1%) and *P. pseudotinctorum* (8.9%) of earlier studies **[23-25]**. The ash content of these lichens was found higher than certain legumes **[26]**.

The content of proteins was higher in case of *P. tinctorum* (11.3%) than that of *R. conduplicans* (5.95%). Proteins are macromolecules composed of amino acids joined by peptide bonds. Proteins play a number of roles in the individuals as structural components, energy source, enzymes, hormones, antibodies, carrier molecules etc. Deficiency of proteins results in several conditions such as kwashiorkor, marasmus etc **[27]**. The protein content of

R. conduplicans of this study was lesser than that of protein content of *R. conduplicans* (9.1%) and *R. hossei* (8.8%) of earlier studies **[23,24]**. In another study of Vinayaka *et al*. **[25]**, the protein content of *P. pseudotinctorum* (16.2%) was found to be higher than that of lichens of this study. Lichens are known to be poor sources for proteins when compared to plants. The protein content of the lichens of this study is shown to be sufficiently lesser than the protein content of pulses **[26,28-30]** and oil seeds **[31,32]**.

In the present study, the crude fibre content of *P. tinctorum* (16.36%) was found higher (more than twice) than that of *R. conduplicans* (5.86%). Crude fibre is the residue remaining after defatting followed by digestion with dilute acid and alkali. Though the fibre content have little food value it is important for proper peristaltic action in the intestinal tract. It contains cellulose, hemicellulose, lignin etc. The fibre imparts a variety of health benefits such as reduced risk of chronic diseases such as diabetes, obesity, cardiovascular disease etc. Fibre also lowers the concentration of low density lipoprotein cholesterol in the blood. Moreover, the fibre eliminates waste from the gastrointestinal tract due to its ability to bind water and thus soften the stool **[22]**. The fibre content of *R. conduplicans* of this study was lesser than that of fibre content of *R. conduplicans* (10.1%) and *R. hossei* (10.8%) of earlier studies **[23,24]**. However, the content of fibre in *P. tinctorum* of this study was found to be higher than that of fibre content of *P. pseudotinctorum* (12%) **[25]**.

The carbohydrate content was found to be higher in *R. conduplicans* (79.08%) than *P. tinctorum* (72.13%). Carbohydrates form an integral part of diet and are important in several ways. They are important as structural components as well as energy source. Carbohydrate supplies energy to cells such as brain and muscle cells. It contributes to fat metabolism and spare proteins as an energy source **[22]**. The carbohydrate content of both lichens was higher than that of carbohydrate content of *R. conduplicans* (61%), *R. hossei* (59.9%) and *P. pseudotinctorum* (53.2%) of earlier studies **[23-25]**. It is known that lichens are rich in carbohydrates. The carbohydrate content of both the lichens is found to be higher than that of carbohydrate content of green gram and certain bean **[29,30]**.

The fat includes triacylglycerols, phosphatidylcholine and cholesterol. Together with proteins and carbohydrates, fats act as major energy source for the body. Fats serve as energy reservoir and yield more energy when compared to other biomolecules. Fats are also important for absorption of certain vitamins such as Vitamin A, D, K and E. Besides, the fatty acids are also significant as they are structural components of cell membranes and precursors for bioactive molecules and have regulatory functions **[22]**. In this study, the crude fat content was found to be higher in *R. conduplicans* (2.1%) than *P. tinctorum* (1.3%). The crude fat content of *R. conduplicans* was lesser than that of fat content of *R. conduplicans* (3.3%) and *R. hossei* (3.2%) of earlier studies **[23,24]**. Similarly, the crude fat content of *P. pseudotinctorum* (6.5%) was higher than that of fat content of both the lichens of this study **[25]**. The crude fat content of the lichens of this study was found to be lesser than that of oil seeds **[31,32]**.

The nutritive value (cal/100g) was shown to be higher in *R. conduplicans* (359.02) when compared to *P. tinctorum* (345.42). The nutritive value of *R. conduplicans* of this study was higher than that of nutritive value of *R. conduplicans* (356.0) and *R. hossei* (348.2) of earlier studies **[23,24]**. Similarly, the nutritive value of *P. tinctorum* was shown to be higher when compared to nutritive value of *P. pseudotinctorum* (336.1) of earlier study **[25]**.

Minerals are inorganic substances found in body tissues and fluids. These represent comparatively smaller portion of the diet when compared to major nutrients *viz*., carbohydrates, proteins and fats. Although these mineral elements do not yield energy they are needed for several metabolic processes that are crucial for life. These mineral elements are classified broadly into major or minor elements based on their daily requirement. The importance of mineral elements is well studied in human, animal and plant nutrition as their deficiencies in the diet often lead to a variety of diseases/disorders **[3,33,34]**. Mineral composition of macrolichens *viz*., *Everniastrum cirrhatum* **[3]**, *Usnea pictoides* **[21]**, *R. hossei* **[24]** and *P. pseudotinctorum* **[25]** from Western Ghats has been studied. In the present study, we estimated the content of 5 major and 6 minor elements in microwave digested lichen materials by ICP-OES technique. ICP-OES technique has an advantage over other analytic techniques being used to estimate mineral elements as the technique can estimate a number of elements at a time. Hence, ICP-OES technique is most widely used for elemental determination and several studies have been performed to validate this technique for metal analysis of a large variety of specimen types including lichens **[21,34,35,36]**.

In the present study, the content of calcium and potassium was highest in *R. conduplicans* and *P. tinctorum* respectively among major elements estimated. The sodium content was least in both the lichens. *R. conduplicans* was shown to contain high calcium when compared to *P. tinctorum*. The calcium content of both the lichens was lesser than calcium content of *U. pictoides* **[21]**. The calcium content of *R. conduplicans* and *P. tinctorum* was higher and lesser respectively than that of calcium content of *E. cirrhatum* **[3]**. The potassium content was high in *P. tinctorum* when compared to *R. conduplicans*. The content of potassium of both lichens was higher than that of potassium content of *R. hossei* **[24]**, *U. pictoides* **[21]**, *E. cirrhatum* **[3]** and *P. pseudotinctorum* **[25]**. The

phosphorus content was high in *P. tinctorum* when compared to *R. conduplicans*. The phosphorus content of both the lichens was high when compared to *R. hossei* **[24]** and *E. cirrhatum* **[3]** while lesser when compared to *P. pseudotinctorum* **[25]**. *P. tinctorum* was found to contain high magnesium than *R. conduplicans*. The magnesium content of both the lichens was higher than that of magnesium content of *U. pictoides* **[21]**. However, the magnesium content of both the lichens was lower when compared to *E. cirrhatum* **[3]**. When compared to *R. conduplicans*, *P. tinctorum* was shown to contain high sodium. The sodium content of *R. conduplicans* and *P. tinctorum* was lesser and higher respectively when compared to sodium content of *U. pictoides* **[21]**.

In case of minor elements, the content of iron and nickel was highest and least respectively in both the lichens. The iron content was found higher in *P. tinctorum* when compared to *R. conduplicans*. The iron content of both the lichens was lesser than that of iron content of *R. hossei* **[24]** and *P. pseudotinctorum* **[25]**. The iron content was higher than that of *E. cirrhatum* **[3]**. The iron content of *R. conduplicans* and *P. tinctorum* was lesser and higher respectively when compared to *U. pictoides* **[21]**. The copper content of both the lichens was lesser than that of copper content of *R. hossei* **[24]** and *P. pseudotinctorum* **[25]** whereas the copper content of lichens of this study was higher than that of *U. pictoides* **[21]** and *E. cirrhatum* **[3]**. *P. tinctorum* was found to contain high zinc when compared to *R. conduplicans*. The zinc content of both the lichens was shown to be higher than that of zinc content of *R. hossei* **[24]** while lesser when compared to *E. cirrhatum* **[3]**, *U. pictoides* **[21]** and *P. pseudotinctorum* **[25]**. The content of manganese of *P. tinctorum* was shown to be far higher when compared to *R. conduplicans*. Manganese content of *R. conduplicans* and *P. tinctorum* was found lesser and higher respectively when compared to *R. hossei* **[24]**, *E. cirrhatum* **[3]**, *U. pictoides* **[21]** and *P. pseudotinctorum* **[25]**. Among minor elements estimated, nickel was detected in least concentration. The content of nickel was high in *P. tinctorum* than that of *R. conduplicans*. The nickel content of *P. tinctorum* and *R. conduplicans* was found higher and lesser respectively when compared to nickel content of *U. pictoides* **[21]**. Likewise, the chromium content was also higher in *P. tinctorum*. When compared to *U. pictoides*, the chromium content of *R. conduplicans* and *P. tinctorum* was lower and higher respectively **[21]**.

CONCLUSION

The proximate and mineral analysis of both the lichens indicates that the selected lichens are nutritionally significant and can be used as food or used in the preparation of the foods. Though poor in protein, the lichens have appreciable carbohydrate content. *P. tinctorum* is rich in crude fibre as well as minerals. The high ash content of *P. tinctorum* is reflected by the appreciable quantity of minerals. Hence, the utilization of these lichens in food may provide nutritive benefit.

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