International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(5): 1003-1007

Received: 18-07-2017
Accepted: 20-08-2017

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Phytochemical screening and proximate composition of lichen *Parmotrema tinctorum* (Nyl.) Hale (Parmeliaceae) from Wayanad, Kerala

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Abstract
Parmotrema tinctorum is an edible lichen used as a spice for flavouring food. In Kerala, southern part of India, its occurrence is reported in the evergreen/sub-tropical forests at an altitude of 750-2300m especially in Wayanad and Idukki districts. Despite large collection from the forests of Kerala for use as spice, the reports on its nutritional composition is meager. In the present study, the phytochemical screening and evaluation of proximate composition of edible lichen *viz*. *Parmotrema tinctorum* (Nyl.) Hale (Parmeliaceae) was carried out. Phytochemical screening of *P.tinctorum* extracts showed presence of carbohydrates, phenols, flavanoids, tannins, terpenoids, coumarins and saponins. The powdered lichen was subjected for proximate analysis to estimate moisture, ash, total fat, total carbohydrate, crude protein and crude fibre. *P. tinctorum* exhibited high content of ash, crude fibre and crude protein indicating the nutritional benefits of this lichen.

Keywords: lichen, *Parmotrema tinctorum*, phytochemicals, proximate composition, Wayanad.

1. Introduction
Lichens are simplest form of plants consisting of a symbiotic association of a fungus (the mycobiont) with a photosynthetic partner (the photobiont), usually either a green algae or cyanobacterium. (Bhattarai, 2008, Sudarshan and Ramachandra, 2010) [1, 2]. They occur in a wide range of habitats throughout the world and are seen as epiphytes on branches of trees in rainforests. It is reported that 50 percent of the all lichen species have antibiotic properties. (Vartia, 1973, Shyam Kumar *et al*, 2010) [3, 4].
The lichens have been used as folk medicine, food, dyes, and as perfumes since ancient times. They produce a wide range of primary and secondary metabolites (Hale, 1983) [5]. Secondary metabolites are called lichen substances, produced mostly by the mycobiont and are distinctive to lichens. Lichen substances show a great range of biological effects, *viz*. antimicrobial, analgesic, antipyretic and antiproliferative and cytotoxic activities (Srivastava *et al*. 2013., Rasmi and Rajkumar, 2014) [6, 7].
Many species of lichens are used as food or as flavourant in the foods. Parmelioid lichens (Lichens belong to Family Parmeliaceae) are available in the market as condiment. *Parmotrema tinctorum* is used as a spice and flavouring agent for meat and vegetables by ethnic groups in India and Nepal (Upreti *et al* 2005) [8]. It is called *Al-Sheba* in Arabic and used as a spice in food, in Arab countries. (Abo-Khatwa *et al*. 1997, Mohaptra, 2011) [9, 10].
Parmotrema tinctorum is prevalent in humid places in the evergreen/subtropical forests between an altitude of 750-2300m above MSL. In Kerala, it was found to be prevalent in areas like Munnar, Nelliampathy, Parambikulam, Siruvani and Wayanad (Kumar *et al.* 2009) [11]. Despite the large collection of this lichen by the tribal people from forests of Wayanad, the reports on its use as a food source are few. Keeping in mind the potential uses of lichen in the preparation of food, and in possible pharmaceutical applications the present study was undertaken to screen the lichen extracts for presence of phytochemicals and to evaluate the proximate composition of lichen *Parmotrema tinctorum* (Nyl.) Hale (Parmeliaceae).

2. Materials and Methods
2.1. Collection and identification of lichen
The lichen *Parmotrema tinctorum* was collected during December 2015 from Wayanad district (2100 MSL), Kerala, India. Sanction for collection of lichen samples has been obtained from

the Department of Forest and Wildlife, Kerala. (Ref.No. WL-10-39996/2015 dt. 19.10.2015). The lichen samples were identified by Jawaharlal Nehru Tropical Botanical Garden and Research institute (JNTBGRI), Thiruvananthapuram. The lichen thalli were cleaned of the substratum and thoroughly washed under distilled water to remove all the debris, dried under shade and powdered.

2.2. Phytochemical screening

The lichen extracts were prepared by cold maceration method using organic solvents viz. petroleum ether, ethyl acetate, acetone and methanol. Five gram of lichen powder was soaked in 25 ml of respective solvent and kept at room temperature for three days. This solution was filtered using Whatman No. 1 filter paper. The filtrate was screened for the given phytochemical constituents (Sazada *et al*, 2009; Raaman, 2006) [12, 13].

i. Test for carbohydrates

Fehling's test: Two ml of extract was added in water and heated with Fehling's solution. Reddish brown colour indicated the presence of carbohydrates.

ii. Test for phenols

To one ml of the extract, added 2 ml of 5% ferric chloride solution along the sides of the test tube. A dark green colour indicated the presence of phenolic compounds.

iii. *Test for flavanoids*

Lead acetate test: Five ml of the extract solution was added with 1 ml of lead acetate solution. Flocculent white precipitate indicated the presence of flavanoids.

iv. *Test for tannins*

Braemer's test: To 2-3 ml of extract, 10% of alcoholic ferric chloride solution was added. Dark blue or greenish grey colouration of solution indicated the presence of tannins.

v. *Test for alkaloids*

Wagner's test: To 3 ml of the extract, 2 ml of Wagner's reagent was added. Reddish brown precipitate indicated the presence of alkaloids.

vi. *Test for saponins*

Foam test: 1 ml of the extract was added to 20 ml distilled water and shaken in a graduated cylinder for 15 minutes. Occurrence of one cm layer of foam indicated the presence of saponins.

vii. *Test for fixed oils*

Two ml of the extract was pressed between two filter papers. Oil smears on the filter papers indicated the presence of fixed oils.

viii. *Test for terpenoids*

Salkowski test: One ml of chloroform was added to 2 ml of the extract followed by a few drops of concentrated sulphuric acid. A reddish brown precipitate produced immediately showed the presence of terpenoids.

ix. *Test for steroids*

One ml of the extract was added with 1 ml of acetic acid, 1 ml of chloroform followed by 0.5 ml H_2SO_4. Change of test solution to blue green colour, denoted the presence of steroids.

x. *Test for quinones*

One ml of the extract was treated with 5 ml concentrated H_2SO_4. Formation of yellow coloured precipitate denotes the presence of quinones.

xi. *Test for coumarins*

Two ml of the extract was treated with 3 ml of 10% NaOH. Yellow colour of the solution indicates the presence of coumarins.

2.4. Proximate composition of lichen

Estimation of moisture content, total fat, total carbohydrate, crude fibre, crude protein, ash content were conducted as per the procedures described by Ranganna (1997) [14], Sadasivam and Manickam (1992) [15].

1. Moisture content

Ten gram of powdered lichen was taken in a pre-weighed silica crucible (W_1) and dried in hot air oven at 100°C, cooled in a dessicator and weighed. The sample was again reheated in oven and cooled until the consecutive weighings did not vary by 3-5 mg; and the final weight was recorded (W_2). Moisture content of lichen was calculated using the formula

Moisture content = $[(W_2\text{-}W_1)$ / weight of material] x100

2. Crude fat

Five gram dry, powdered lichen material was taken in a thimble and was extracted using petroleum ether in a Soxhlet extraction apparatus for six hours. The apparatus was cooled and the solvent containing fat was drained into another pre-weighed (W_1) container; and was evaporated on water bath until no odour of solvent left. After cooling in a desiccator, the container was again weighed (W_2). Heating and weighing was continued until constant weight was recorded. The crude fat (%) was calculated using the formula:

Crude fat (%) = [Weight of fat $(W_2\text{-}W_1)$ / Weight of sample] ×100

3. Crude fibre

Two gram dry, fat-free lichen powder (residue from fat estimation) was boiled with 200 ml of 1.25% H_2SO_4 for 30 minutes. The content was filtered and the residue was washed with boiling water to clear off the acid. The residue was further boiled with 200 ml of 1.25% NaOH for 30 minutes. The mixture was filtered again, washed with 25 ml of boiling 1.25% H_2SO_4, and then with three 50 ml portions of water and finally with 25 ml ethyl alcohol (95%). The residue was transferred to a pre-weighed silica crucible (W_1), dried for two hours at 130 ^{0}C in hot air oven. The crucible was cooled in a desiccator and weighed (W_2). It was heated in a muffle furnace at 600 ^{o}C for 30 minutes and the crucible was again cooled and weighed (W_3). Crude fibre content was calculated using the formula:

Crude fibre % =Loss in weight $[(W_2\text{-}W_1)\text{-}(W_3\text{-}W_1)]$ / weight of sample x100

4. Crude Protein

The protein content of lichen was estimated by the micro Kjeldahl method. Two gram of lichen powder in a Kjeldahl flask was added with 30ml of concentrated H_2SO_4, 10g potassium sulphate and 1 g copper sulphate. The mixture was heated gently first and then strongly once the frothing had ceased. The colourless solution was heated for another hour, cooled and diluted to 100 ml with distilled water in a volumetric flask. Ten ml of aliquot of the digest was measured into the decomposition chamber of the distillation

apparatus and 10 ml of 40 % NaOH was added and the ammonia released was trapped into 10 ml of 2 % boric acid solution containing mixed indicator. The boric acid-ammonia solution was titrated against 0.1N H_2SO_4. The percentage of nitrogen and crude protein was calculated by the formulae:

% Nitrogen = T x N x 0.0014 x D x 100 / Weight of the sample x V, where T is titrate value of sample, N is normality of H_2SO_4, 0.0014 is milliequivalent weight of nitrogen, D is dilution of sample after digestion, V is volume of sample taken for distillation

Crude protein = % nitrogen X 6.25, where 6.25 is correction factor.

5. Total carbohydrate

The carbohydrate content was measured by anthrone method. Fifty milligram *P.tinctorum* powder was hydrolysed by 2.5 N HCl over boiling water bath for three hours. The mixture was cooled and neutralized with $NaCO_3$ granules; then made upto known volume and filtered. This filtrate was pipetted into test tubes and made up the solution to one milliliter with distilled water. To the tubes, 4 ml anthrone reagent was added and heated in boiling water bath for eight minutes. The contents were cooled rapidly and the absorbance of green complex was measured at 630 nm in UV-visible spectrophotometer (UV-1800 Shimadzu, Japan). The content of carbohydrate in the lichen sample was found out by preparing a standard curve using different concentrations of glucose.

Amount of carbohydrates present in 100 mg sample= mg of glucose/volme of test sample x100.

6. Total ash

Five gram of lichen powder was taken in a pre-weighed crucible (W1). The crucible was heated over a low flame till the sample was completely charred, and the carbon residue was heated in a muffle furnace at 600°C for about 3–5 hours. The crucible was cooled in a desiccator and weighed (W2). This process was repeated till the weight of ash become constant. Total ash content was calculated by the formula:

Total ash content = [weight of ash (W_2-W_1) / weight of material] x100

2.5. Data analysis

All the analyses were done in triplicates. The results were analysed using SPSS (USA) version 16.0 for Windows and expressed as mean±SD.

3. Results and Discussion

Parmotrema tinctorum is a foliose lichen which is mostly corticolous (grows on bark) in habit. The habitat of the lichen are moist or shady places in the evergreen/sub tropical forests between an altitude of 750-2300m. Thallus is foliose, loosely attached, gloucous grey; lobes rotund, upto 20 mm wide, margin entire, upper surface smooth, shining, emaculate, sometimes cracked; lower surface black, rhizines sparse, and marginal area brown. (Kumar, 2000) [16]. The lichen *Parmotrema tinctorum* is shown in Fig.1.

Fig 1: *Parmotrema tinctorum*

3.1. Phytochemical screening

Phytochemicals are natural bioactive compounds found in plants, act as an defense system to protect against biotic and abiotic stress conditions. Phytochemicals are divided into primary and secondary constituents; based on their functions in plant metabolism. Primary constituents include common sugars, amino acids, proteins whereas secondary constituents consist of alkaloids, terpenoids, flavanoids and phenolic compounds (Krishnaiah *et al*., 2007) [17]. Flavonoids play vital role in protecting biological systems against the harmful effects of oxidation on macromolecules, such as carbohydrates, proteins, lipids and nucleic acids. Thus they have been reported to possess properties, such as anti-inflammatory, antimicrobial, oestrogenic, anti-allergic, antioxidant, and cytotoxic antitumor activity (Saxena *et al*., 2013) [18]. Saponins are glycosides of triterpenes and sterols and are used as expectorant and emulsifying agent. (Belewu *et al*, 2009) [19]. According to the study by Rashmi and Rajkumar (2014) [7], saponins were detected only in one lichen species *Usnea subflorida*, not in *Parmotrema tinctorum*. But in the present study, petroleum ether extract had shown the presence of saponins.

The results of preliminary phytochemical screening for secondary metabolites are presented in Table 1. Methanol extract contained maximum phytochemicals *viz*. carbohydrates, phenols, flavanoids, tannins, terpenoids, fixed oils and coumarins. Methanol being highly polar dissolves most of the secondary metabolites of *Parmotrema tinctorum* as reported by Rashmi and Rajkumar (2014) [7]. In this study, extracts using medium polar solvents *viz*. acetone and ethyl acetate was found positive for carbohydrates, phenols, tannins, and terpenoids, which were not reported in the study conducted by Rashmi and Rajkumar (2014) [7]. Acetone extract also showed presence of fixed oils; whereas ethyl acetate indicated presence of flavanoids. Petroleum ether extract did not show any phytochemicals except saponins. Presence of alkaloids, steroids and quinones was not detected in all the four extracts.

Lichen substances are insoluble in water and hence extracted using organic solvents (Tiwari *et al*., 2011) [20]. Most lichen substances with antibiotic activity are phenolic compounds e.g. usnic acid (Hale, 1983) [5]. The phenolic compounds *viz*. lecanoric acid and atranorin are the main secondary metabolites detected in *P. tinctorum* which show biological activities (Din *et al*., 2010, Sebastian *et al*., 2014) [21, 22].

Table 1: Phytochemical screening of *P. tinctorum* with different solvents

	Extracts			
Phytochemicals	**Methanol**	**Ethyl acetate**	**Acetone**	**Petroleum ether**
Carbohydrates	+	+	+	-
Phenols	+	+	+	-
Flavanoids	+	+	-	-
Tannins	+	+	+	-
Alkaloids	-	-	-	-
Saponins	-	-	-	+
Fixed oils and fats	+	-	+	-
Terpenoids	+	+	+	-
Steroids	-	-	-	-
Quinones	-	-	-	-
Coumarins	+	-	-	-

+ Presence of compound

- Absence of compound

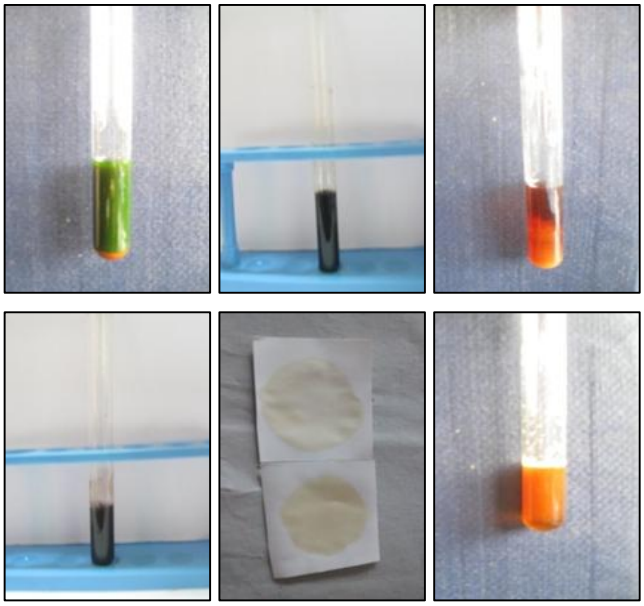

Fig 2: Presence of phytochemicals in the methanol extract a) carbohydrates b) phenols c) flavanoids d) tannins e) fixed oil f) coumarins

3.3. Analysis of proximate composition

The proximate composition of *P.tinctorum* was evaluated and the results were shown in Table 2. Total ash, crude fibre and crude protein content of *P. tinctorum* were found to be high.

Table 2: Proximate composition of *P. tinctorum*

Parameter	Content
Moisture (%)	8.09 ±0.64
Total carbohydrates (g/100g)	20.03±0.61
Total ash (%)	10.50 ±0.34
Crude fibre (%)	14.16±2.11
Total fat (%)	1.28±0.152
Crude protein (%)	15.70±1.30

Each value represents the mean ± SD of three determinations

The moisture content of *P. tinctorum* lichen was found to be 8.09 %. The moisture content of *P. tinctorum* was reported to be 9.12% (Kambar *et al*., 2014) [23] and 9.13% (Raj *et al*, 2014) [24]. Lichens are poikilohydric organisms and are able to survive long periods of dry conditions in a dormant stage. They lack stomata, cuticle and any water storage system. Water vapor is lost readily from the whole surface of lichens (Lumbsch, 2008) [25].This may be the reason for low moisture content of lichen. Low moisture food materials are less susceptible to microbial damage (Ramesh, 2011) [26].

Carbohydrates function as source of energy and as structural components (Chavan and Patil, 2015) [27]. It is the major component of lichen species viz. *P. tinctorum* (72.13%) *R. conduplicans* (79.80%), *Ramalina hossei* (59.9%) and *Parmotrema pseudotinctorum* (53.2%) as reported by Kambar *et al* (2014) [23] by difference method (carbohydrate=100-ash+moisture+fat+protein). But on analysis by UV-vis spectrophotometry in the present study, which is a specific method, the carbohydrate content was found to be 20.03g /100 g. This is in concordance with report by Lal and Rao (1956) [28] who observed the carbohydrate content of *P.tinctorum* to be 25.0 g/100 g. Raj *et al* (2014) [24] also reported a similar value for quantity of carbohydrate (32.35%) in *P.tinctorum*.

The ash content represents the mineral content of organic samples (Ooi *et al*, 2012) [29]. The ash content of *P. tinctorum* lichen was found to be 10.50 %. A high ash value suggests that the lichen could contain high percentage of mineral matter. This is in accordance with study by Raj *et al*, (2014) [24] in which they reported an ash content of 11.65 % for *P. tinctorum.* Earlier reports of Lal and Rao (1956) [28] also showed a high ash value for this lichen to the tune of 12.6%. Another edible lichen *Ramalina conduplicans* also recorded a significant ash content of 10.0% (Vinayaka *et al*, 2009) [30].

The present study revealed high crude fibre content for *P. tinctorum* (16.36%). Crude fibre is the residue remaining after defatting followed by digestion with dilute acid and alkali. Cellulose and lignin form major part (97%) of crude fibre. Insoluble fiber passes through the intestines undigested, absorbing water and organic toxins (Chavan and Patil, 2015) [27]. The crude fibre values reported for rice was only 0.2% whereas Bengal gram has 25.6% crude fibre content, while green leafy vegetables contain 2.9 – 4.0% (Kamath and Balavedy, 1980) [31].

Phytochemical screening of extracts showed the presence of fixed oils. On analysis, *P. tinctorum* recorded low fat content to the tune of 1.28%. This agrees with other reports where *P. tinctorum* showed a fat content of 1.3% (Kambar *et al*, 2014) [23], 1.8% (Raj *et al*, 2014) [24]. The crude fat content of *P. tinctorum* was found to be comparable to other spices like nutmeg mace (1.6%), fresh ginger (0.9%) and cardamom (2.2%) (Gopalan *et al*, 2009) [32].

P. tinctorum was found to have a high crude protein content of 15.70% on analysis. Lal and Rao (1956) reported similar protein content for *P. tinctorum* (13.8%). According to Behadur *et al* (2015) [33] certain lichen species recorded high crude protein, content and thus have good food value viz. *Dermatocarpon moulnsii* (20%), *Lobaria isidiosa* (20%), *Roccella montagnei* (14%) and *Parmotrema tinctorum* (14%). Kambar *et al*. (2014) [23] also observed a higher protein content of 11.3 % in this species.

Conclusion

The phytochemical screening and proximate analysis revealed the nutritional significance of lichen *Parmotrema tinctorum*. *P. tinctorum* extracts showed presence of phytochemicals *viz*. carbohydrates, phenols, flavanoids, tannins, terpenoids, saponins and coumarins which may be the basis of it's biological properties. The lichen *P. tinctorum* is rich in crude fibre and crude protein. High ash value reflects its abundant mineral matter. Apart from flavouring purpose, this lichen shows potential for production of nutraceuticals.

Acknowledgements

Authors thank Dr. Biju. H., Microbiologist, Jawaharlal Nehru Tropical Botanical Garden and Research institute, Thiruvanathapuram, Kerala for identification of lichen.

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