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Subcritical Fluid Extraction of Macro Lichens, Optimization and Phytochemical Screening

Pradeep M J[1], Shivabasappa K L[2]*

[1]Department of Chemical Engineering, Siddaganga Institute of Technology, Main Road, Bengaluru - Honnavar Road, Tumakuru, Karnataka 572103, India
[2]Sapthagiri College of Engineering, 14/5, Hesaraghatta Main Road, Chikkasandra, Jalahalli West, Bengaluru, Karnataka 560057, India

***Author for Correspondence:** Dr. K.L.Shivabasappa, Principal, Sapthagiri College of Engineering, 14/5, Hesaraghatta Main Road, Chikkasandra, Jalahalli West, Bengaluru, Karnataka 560057. E-mail:pradeep.jayappa@gmail.com, Phone: +91-9164460343

Abstract: ***In the present study, we made an effort to screen the novel bioactive compounds from macro lichens using subcritical fluid extraction method. The lichens of genus namely Flavoparmelia caperata, Pamtrema pseudotinctorum, Ramalina hossei, P. grayanum, Heterodermia comosa, Physcia aipolia and Roccella montagneig collected from Western Ghats regions of Shivamogga district, Karnataka were used in our study. Based on the availability and abundance of lichen material, two macro lichens viz., P. pseudotinctorum and R. hossei were selected for Sub-critical Fluid Extraction (SCFE). In optimization of SCFE process was noticed that, at 60 bar to 70 bar pressurethe gradual increase of extract from 2.60 % to 3.18 % in P. pseudotinctorum and 2.83 %to 3.39 %R. hossei respectively. In-case of time of interaction gradual increase in the weight of extract from 2.31 g to 3.13 % and 2.63 % to 3.29 % in case of P. pseudotinctorum and R. hossei respectively at 60 minutes. The temperature shows positive influence on the yield, gradual increase in temperature range from 30 to 40 °C the amount extract was increased from 2.22to 5.94 % and 2.45 to 6.22 %for the P. pseudotinctorum and R. hossei respectively. Preliminary phytochemical analysis of sub-critical fluid extracts of lichens revealed the presence of tannins and absence of flavonoids and saponins. Steroids were detected in P. pseudotinctorum while terpenoids, alkaloids, glycosides were detected in R. hossei. Among lichens, high carbohydrate content (59.9 %) was observed in R. hossei than P. pseudotinctorum (53.2 %). Nutritive value (cal/100 g) was found to be greater than 300 cal/100 g in both the lichens. It was found to be higher in case of R. hossei than P. pseudotinctorum. Phosphorus was found to be higher in case of P. pseudotinctorum while potassium content was same in both the lichens. In case of microelements, P. pseudotinctorum was rich in Fe, Mn, and Zn. R. hossei was found to possess high concentration of Cu.***

Keywords: Subcritical Fluid Extraction, Conventional Extraction, Phytochemical screening, Optimization

1. Introduction

At present, plant medicine comes from plant extraction, has occupied nearly 30 percent to 40 percent among the thousands of worldwide used pharmaceutical products [1]. Chemical composition of various medicinal plants is very complex, usually containing many kinds of effective ingredients [2]. Numerous methods, including conventional solvent extraction, steam distillation, and sublimation, etc., are known for extracting phytochemicals from plant materials, most based on sequential extraction processes incorporating one or more organic solvents in combination with washing steps [3]. While such methods are useful for extraction and purification of small quantities of phytochemicals for research purposes, they are difficult to scale to commercial through-put volumes because of the problems associated with cost-effectively, safely and completely removing and recovering the organic solvents from the extracts and spent plant materials [4].

In recent years, many new technologies and methods, such as ultrasonic extraction, microwave extraction, membrane separation technology, molecular distillation, macro porous resin adsorption and supercritical CO_2 extraction technology, are introduced to the extraction and separation of effective components in medicinal plants. On the other side a new extraction technique, “subcritical extraction”, has emerged [5-7].

Lichens are the composite organisms from algae or cyanobacteria living among filaments of multiple fungi. The combined lichen has properties different from those of its component organisms. Recently, much attention has been paid to biological activities of lichen metabolites. The lichen extracts and the purified metabolites are known to exhibit a wide array of bioactivities such as antibacterial, antifungal, antioxidant, antitumor, anti-herbivore, phytotoxic, analgesic, antipyretic, wound healing, anti-termite, enzyme inhibitory, anti-inflammatory, insecticidal and others [8,9].

The secondary metabolites from lichens can be extracted using conventional extraction techniques like aqueous extraction and soxhlet extraction. These methods were inefficient because they produced low and impure yield. The more efficient way of extracting metabolitesfrom medicinal plants is by novel extractions using subcritical method using different solvents. Solid liquid extraction is a process in which a solid material is contacted with a liquid solvent for the removal of one or more constituents of the solid material. In this process, a solid phase contacts a liquid phase and mass is transferred from the solid phase to the liquid phase. The solid to be leached must be finely divided so that the liquid solvent contacts all parts of solid. Usually, the desired solute is soluble while the undesired component is insoluble in the solvent used.

Subcritical fluids based technologies are involved inwide variety of industrial applications which have shown significant progress in recent years. Many industrial

sectors are concerned including food, cosmetics, pharmaceutics, materials, chemistry, energy and waste treatment. The related supercrical fluid processes include extraction, impregnation, formulation, sterilization, cleaning, energy and waste treatment among others.After extraction the second step in phytochemical analysis involves isolation and identification of phyto-constituents. Due to the fact that plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. Plant extracts are subjected to phytochemical screening involving various chromatographic and spectroscopic methods [10].

Novel techniques offers a great opportunity for developing countries those have potential in the development of their herbal medicines as an important industry. The development of herbal product including medicinal plants and essential products and spices for export can help to increase income among farmers, reduce poverty and stimulate entrepreneurship and create a favorable business environment to integrate into the global market place.

2. Materials and Methods

2.1. Collection and identification of lichen

The lichen specimens were collected with their ecological notes, which includes; the host tree type, location of the lichen thallus (trunk, branch, twigs, leaves, soil or rock substratum). The collected specimens were investigated morphologically, anatomically and chemically for further identification (Kumar, 2009b) [11].

2. 2. Extraction of secondary metabolite from lichens by SCFE and Conventional Extraction

2.2.1. Preparation of lichen extracts

Seven species of lichens were selected for further study based on their availability and abundance. The selected lichens were washed 2-3 times in distilled water and dried at room temperature under shade. After drying, the lichen material were ground to fine powder and used for the extraction of secondary metabolites by aqueous method, soxhlet method, microwave method and sub critical extraction [12].

2.2.2. Sub critical Fluid Extraction

Extraction was carried out in JASCO 900 series Subcritical fluid extractor using CO_2 in a subcritical state. The SCE system and components were acquired from JASCO (Japan Spectroscopic Co.) 900 series. The Subcritical fluid extractor included the following: 100 mL extraction vessel, temperature control unit (JASCO C0-965), high-pressure pump (JASCO-PU-980) and automated back pressure regulator (JASCO 880-81).

2.2.2.1. Packing of Extraction vessel

The extraction vessel was rinsed and cleaned with acetone and dried. A small piece of ceramic wool was placed at the bottom of the vessel. The powdered plant material (50g) was mixed well with 2.0mm diameter glass beads and placed in the extraction vessel and adjusted to maintain a proper flow rate of CO_2 in the extractor (Chemat *et al*., 2004; Wang and Weller, 2006) [13, 14].

2.3. Optimization of SCFE parameters

In optimization of SCFE conditions, each extraction parameter viz., extraction pressure, extraction temperature, CO_2 flow rate and co-solvent percentage was optimized separately; one being tested was varied keeping the other parameters constant.

Dynamic extraction time was kept constant for 1h for the study of each parameter.

1. Parameter 1: Temperature study: Effect of temperature on extraction was studied in the range of 15°C to 40°C at an interval of 5°C, with constant pressure of 60bar and CO_2 flow rate at 12.0mL/min. Dynamic extraction time was kept constant for 1h for the study of each parameter.
2. Parameter 2: Pressure study: Effect of pressure on extraction was studied in the range of 10 bar to 70 bar by setting optimum temperature at 35°C and CO_2 flow rate at 12.0mL/min.
3. Parameter 3: CO_2 flow rate study: The next step was to determine the optimal CO_2 flow rate for extraction. The different flow rate values set were from 2.0mL/min to 12.0mL/min by keeping optimum temperature at 35°C and optimum pressure at 60 bar.
4. Parameter 4: Extraction time study: Effect of time on extraction was studied in the range of 30 min to 150 min by setting optimum temperature at 35°C, pressure at 60 bar and CO_2 flow rate at 12.0mL/min.
5. Parameter 5: co-solvent percentage study Murga *et al.*, (2000) [15], Cavero *et al*., (2006) [5].

2.4. Design of Experiment

Subcritical extraction depends on different factors and despite of its multiple advantages, also because of the difficulties and test performance complexities in high pressure and in order to reduce the number of tests, it is necessary to design of the experiment(DOE).The most important factors concerning the total yield of extraction and the concentration of the essence in the subcritical fluid are: pressure and the temperature of the extraction, flow rate or the velocity of subcritical fluid and the time of the test or dynamic time.

Table 1: Variable parameter range in SCF for Lichens

Sl. No.	Parameters	Units	Variable Range
1	Pressure	Bar	20-70
2	Temperature	°C	15-40
3	Time	Min	30-60
4	Flow rate	mL/min	2-12
5	Co-solvent concentration	%	5-15

2.5. Determination of Extraction yield

Ethanolic SCF extracts obtained under optimized SCFE conditions were collected and pooled together. The pooled extract was filtered through whatman filter paper no. 1 to remove solid particles, if any. The filtered extract was then completely dried in the oven at 40°C and the final constant weight was recorded and calculated

$$Y_{extract} = \frac{m_{extract}}{m_{herb}} 100 \quad \ldots (2.1)$$

Where, $Y_{extract}$ is the % extraction yield, $m_{extract}$ is the crude extract mass (g) and m_{herb} is the extracted herb mass (g).

2.6. Phytochemical Screening of the Lichen material

2.6.1. Proximate Analysis

Various proximate parameters namely moisture, ash, crude fiber, crude fat, protein and carbohydrate content were analyzed in the dried and powdered lichen materials.

2.6.2. Determination of Elemental Composition of Selected Lichens

The concentration of macro elements namely Potassium (K) and Phosphorus (P) and microelements namely Copper (Cu), Manganese (Mn), Iron (Fe) and Zinc (Zn) was estimated using atomic absorption spectrometer.

2.6.3. Qualitative analysis of Phytochemicals

Phytochemical tests are done in plant extracts for the recognition of presence of different chemical constituents such as; alkaloids, glycosides, phenolic compounds, flavonoids, essential oils, carbohydrates, proteins, steroids, saponin glycosides, tannins and other substances which are accountable for the biological activity. Thus the chemical experiments are carried out in the various solvent extract for the recognition of different chemical constituents [16].

3. Results and Discussion

3.1. Plant material collection and identification

The lichens namely *Flavoparmelia caperata, Pamtrema pseudotinctorum, Ramalina hossei, P. grayanum, Heterodermia comosa, Physcia aipolia and Roccella montagnei* grown in and around the areas of Shivamogga and Davanagere were used as objects to the study.

The presence of secondary metabolites in lichen materials was detected using thin layer chromatography. Thin layer chromatography (TLC) in solvent A (180 mL toluene: 60 mL 1-4, dioxine: 8 mL acetic acid) was completed to identify secondary metabolites by means of standard techniques. Metabolites namely atranorin and lecanoric acid were detected in *P. pseudotinctorum*. *R. hossei* was shown to possess usnic acid and sekikaic acid. In case of lichen *Flavoparmelia caperata* found to be usnic acid and atranorin. The presence of Atranorin was detected in-case of *Physcia aipolia, Parmotrema grayanum* and *Roccella montagnei* (Table-2).

Table 2: Secondary metabolites detected in selected lichens using TLC

Metabolite	*P. pseudotinctorum*	*R. hossei*	*F. caperata*	*H. comosa*	*P.aipolia*	*P.grayanum*	*R. montagnei*
Usnic acid	-	+	+	-	-	-	-
Atranorin	+	-	+	+	+	+	-
Lecanoric acid	+	-	-	-	-	-	+
Salanizic acid	-	-	-	-	-	-	-
Sekikaic acid	-	+	-	-	-	-	-

Lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world. They produce characteristic secondary metabolites that are unique with respect to those of higher plants (Lawrey**,** 1986) [17].Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, anti-proliferative and cytotoxic effects (Muller, 2002)**[**18].

3.2. Conventional extraction and yield of the components

The yield of extract in aqueous and soxhlet extraction techniques with methanol, Petroleum ether and ethyl acetate as solvents. The yield of extract was very much negligible in case of aqueous extraction, whereas in soxhlet extraction process methanolic extract shown appreciable yield compared to petroleum ether and ethyl acetate extracts. The lichens *P. pseudotinctorum and R. hossei* exhibits more yield compared to other lichens.

3.3. Sub-critical extraction (SCE)

3.3.1. Process optimization using CO_2 as solvent

The effect of parameters like Temperature, pressure, solvent flow rate and extraction time on yield of extraction was studied for both the macro lichens. At the end of each run, the total amount of produced essence mass on the mass of consumed feed powder of Lichen by subcritical extraction.

3.3.1.1 Pressure as variable at constant temperature and flow rate

The influence of the operating pressure on the process was examined at isothermal conditions. The operating pressure of the extraction fluid was being altered in the range from

10 to 70 bar by application of the "step-wise" technique and at a constant operating temperature of 35°C and solvent flow rate of 12 mL/min. By gradual increase in pressure the amount of extract was increased from 2.60 to 3.18 % in case of *P. pseudotinctorum* and 2.83 to 3.39 % in case of *R. hossei*. The maximum extract was observed at 60 bar to 70 bar pressures for *P. pseudotinctorum and R. hossei* respectively**,** so the further experiments were carried out at 60 bar pressure.

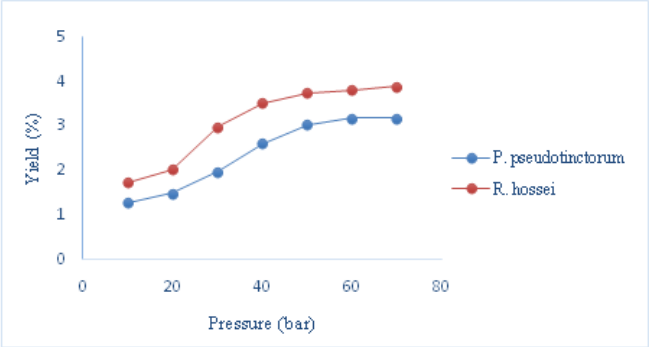

Figure 1: Effect of varying pressure in extraction of secondary metabolites by sub- critical fluid extraction by maintaining constant temperature and solvent flow rate.

3.3.1.2. Temperature as variable at Constant Pressure and flow rate

Feed of 50g for variable temperatures of 40, 50, 60, 70 and 80°C at Pressure of 60 bar and solvent flow rate of 12 mL/min was maintained for extraction. Solvent temperature was increased for each run of the process. Regarding the temperature's positive influence on the yield (Fig.4.3), the range from 30 to 40 °C is of particular importance and interest due to the fact that this positive influence is accompanied by the adequate yield increase resulting from the increase of the vapour pressure. Actually, further experiments were guided at a constant temperature of 35 °C.

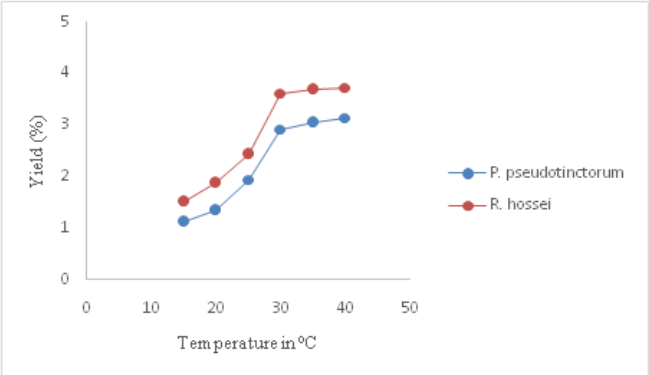

Figure 2: Effect of varying temperature in Extraction of Secondary Metabolites by Sub-critical Fluid Extraction by maintaining constant pressure and solvent flow rate.

3.3.1.3. Flow rate as variable at constant temperature and pressure

In order to determine the influence of the extraction agent' (CO_2) operating flow rate on the yield following experiments were conducted at constant values of operating pressure, operating temperature, extraction time, while the operating flow rate of the extraction agent (CO_2) was altered in the range 2-12 mL/min with a regular interval of 2 mL/s at a pressure of 60 bar and temperature of 35°C was maintained for extraction. As the time of interaction was gradually increased the weight of extract was increased 2.31 to 3.13 % and 2.63 to 3.29% in case of *P. pseudotinctorum* and *R. hossei* respectively.

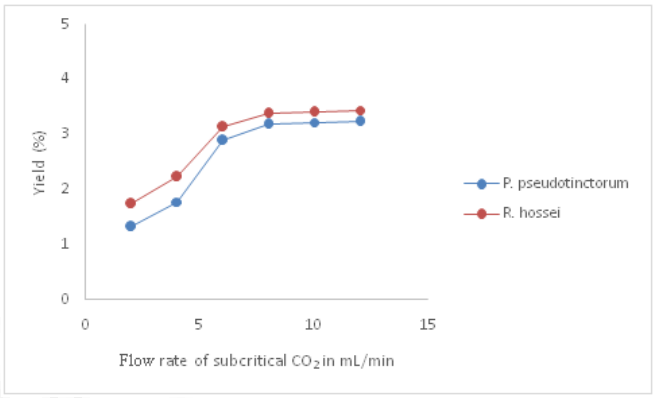

Figure 3: Effect of varying solvent flow rate in extraction of secondary metabolites by sub-critical fluid extraction by maintaining constant pressure and temperature

3.3.1.4 Effect of co-solvent on extraction

Fig. 4 exhibits the variation in the extraction yield by the addition of co-solvent or entrainer. In the present study, an Ethanol range from 5% to 15% was added along with solvent. The best extraction yields was achieved at 35ºC temperature, 60 bar pressure and 12 mL/min solvent flow rate using 9 to13% ethanol as entrainer.

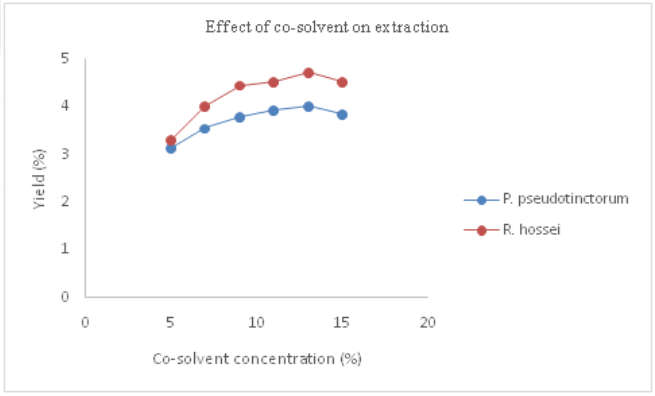

Figure 4: Effect of co-solvent on extraction of secondary metabolites by sub critical fluid extraction by maintaining constant pressure, temperature and flow rate

3.3.1.5 Influence of the granulation of the raw material on the subcritical extraction process

Earlier reports indicate that the variation of the lichen granulation has no great influence on the yield of total extract. Although, at smaller granulation of the material, slightly higher values of yield were recorded which is in accordance to the Fick's law of mass transfer as smaller granulation of the material provides greater mass transfer contact surface for the extraction fluid and the raw material. When analyzed systematically, going from

smaller to bigger granulation as well as focusing on mixed granulation fraction, experimental results indicate a minor difference for yield percentage values. This is due to the symbiotic nature of the lichen, algae - fungus, that provides specific physical constitution. Namely, algae have a specific "threadlike" constitution and the granulating process of the lichen produces "threadlike" shaped particles that have only minor influence on the total yield at higher operating conditions.

3.3.1.6 Process optimization using R134A as solvent

The effect of parameters like Temperature, pressure, solvent flow rate and extraction time on yield of extraction were studied for both the macro lichens. At the end of each run, the total amount of produced essence mass on the mass of consumed feed powder of Lichen by subcritical extraction with R134a as solvent was summarized in table and graph.

3.3.1.7 Influence of extraction time on the yield of extraction

The functional dependency of total yield from the extraction time in Fig.5 presents the subcritical extraction process dynamics. In the frames of this investigation the interpretation of the results indicates a desired range from 2 to 3 hours, as the process enters equilibrium state. The results were observed to be great at 60 min for both the samples.

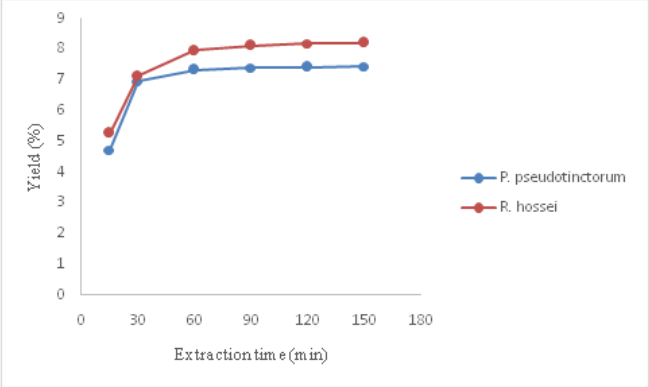

Figure 5: Effect of on extraction of secondary metabolites by sub-critical fluid extraction by maintaining constant pressure, temperature and solvent flow rate

3.3.1.8 Phytochemical screening of selected lichens

Preliminary phytochemical analysis of sub-critical fluid extracts revealed the presence of tannins in sub-critical fluid extracts of both the lichens tested. Steroids were detected in *P. pseudotinctorum* while terpenoids, alkaloids, glycosides were detected in *R. hossei*. Phytochemicals namely flavonoids and saponins were not detected in sub-critical fluid extracts shown in Table-3.

Table 3: Phytochemical constituents detected in sub-critical fluid extraction of lichens.

Metabolite	*P. pseudotinctorum*	*R. hossei*
Tannins	+	+
Flavonoids	-	-
Alkaloids	-	+
Steroids	+	-
Glycosides	-	+
Saponins	-	-
Terpenoids	+	+

3.3.1.9 Proximate analysis

The proximate composition of lichens selected in this study is shown in Table 4. Among lichens, *R. hossei* was found to possess high moisture content (16 %) than *P. pseudotinctorum* (15.2 %). Ash content was high in *R. hossei* (12.1 %) while it was found to be 8.9 % in *P. pseudotinctorum*. Advantage of *P. pseudotinctorum* lies in its comparatively high crude fibre (12 %) and protein content (16.2 %). *P. pseudotinctorum* was found to possess fat content of 6.5 % which is quite high when compared to *R. hossei*. Among lichens, high carbohydrate content (59.9 %) was observed in *R. hossei* than *P. pseudotinctorum* (53.2 %). Nutritive value (Cal/100 g) was found to be greater than 300 cal/100 g in both the lichens. It was found to be higher in case of *R. hossei* than *P. pseudotinctorum*.

Table 4: Proximate composition of selected lichens

Proximate parameter	*P. pseudotinctorum*	*R. hossei*
Moisture (%)	15.2	16.0
Ash (%)	8.9	12.1
Fibre (%)	12.0	10.8
Protein (%)	16.2	8.8
Fat (%)	6.5	3.2
Carbohydrate (%)	53.2	59.9
Nutritive value (Cal/100g)	336.1	348.2

All human beings require a number of complex organic compounds as added caloric requirements to meet the need for their muscular activities. The increasing populations of the world food demands have overwhelmed the available land resources. It has been reported that protein-calories malnutrition deficiencies is a major factor responsible in nutritional pathology (Roger *et al*, 2005) [19]. The dietary fibre plays an important role in decreasing the risks of many disorders such as constipation, diabetes, cardiovascular diseases, obesity etc. (Spiller, 2001) [20].

The carbohydrates are main source and store of energy. They are the starting substances for biological synthesis of many compounds. The trace elements, together with other essential nutrients, are necessary for growth, normal physiological functioning, and maintenance of life. They must be supplied in the food, since the body cannot synthesize them. Some of them are vitally important for health. Recommended intakes have been set for some trace elements and their deficiency can lead to disease (Janab and Thompson, Reddy, 2002) [21]. Cu is a component of many enzyme systems such as cytochrome oxidase, lysyl oxidase and ceruloplasmin, an iron-oxidizing enzyme in blood (Mills, 1981) [22]. The observation of anemia in Cu deficiency may probably be related to its role in facilitating iron absorption and in the incorporation of iron

into hemoglobin (FAO/WHO, 1974) [23]. Zn is a component of many metallo-enzymes, including some enzymes which play a central role in nucleic acid metabolism (Atukorala and. Waidyanatha, 1987) [24].

In addition, Zn is a membrane stabilizer and a stimulator of the immune response (Hambidge1981) [25].Its deficiency leads to impaired growth and malnutrition (Prasad1981) [26]. Manganese (Mn) is essential for hemoglobin formation but excess is harmful (Critchley1986) [27]. Na and K take part in ionic balance of the human body and maintain tissue excitability. Because of the solubility of salts, Na plays an important role in the transport of metabolites. K is of importance as a diuretic (Indrayan *et al*, 2005) [28]. Many lichens have been used medicinally across the world.

A lichen's usefulness as medicine probably usually comes from the lichen secondary compounds that are abundant in most lichen thalli. Lichens are most commonly used for medicine, dye, or food. As a food stuff lichens have often been directly eaten by humans, but they have also been used indirectly to make alcohol or molasses, or to feed to livestock. The nutrient composition of lichens varies widely between different species of lichens but they are generally high in carbohydrates and low in most other nutrients. It was found that calcium and iron levels are higher in lichens than cereals and are more comparable to green leafy materials. Lichens are one of the lesser-known nutritive sources to reduce the malnourishment problems in most of the countries. The results of this study highlighted the importance of the macro lichens in terms of their rich carbohydrate, protein, crude fibre and mineral composition and justify their possible use in reducing problems associated with malnourishment.

Table 5: Elemental Composition of Selected Lichens

Lichens tested	Elemental composition					
	Macro elements (%)		Micro elements (ppm)			
	P	K	Fe	Mn	Zn	Cu
P. pseudotinctorum	0.097	0.080	19573	123.5	74.1	114
R. hossei	0.065	0.080	10358	34.7	32.8	180

Elemental composition of lichens tested showed varied results. Phosphorus was found to be higher in case of *P. pseudotinctorum* while potassium content was same in both the lichens. In case of microelements, *P. pseudotinctorum* was rich in Fe, Mn, and Zn. *R. hossei* was found to possess high concentration of Cu as shown Table-5.

4. Conclusion

In the present research we come-out with efficient extraction method i.e. subcritical fluid extraction to isolate novel components from macro lichen *R.hossei.* The SCF extraction method gives more yield, purified product, less consumption of solvents as well as to reduce the time required for extraction and the technology replace the present conventional methods as the extraction technologies will be safe in all circumstances.

In our study, extraction of novel compounds from macro lichens was undertaken using SFE by stimulation of yield through the entrainers along with solvents to boost the extraction process. At present, the uses of conventional methods are more economic due to the use of more solvent and requirements of more raw materials as well time-durable. Hence the present study approached for the cost effective, eco-friendly effective with more reliable extraction technology which can be used in safer mode.

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