Phytochemical screening, phenolic contents and reducing power assay of selected lichens from Deosi National Park, Pakistan

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***Abstract*-** Lichens are a complex life form that is a symbiotic partnership of two distinct organisms, a fungus, and an alga. This study aimed to investigate phytochemical screening, phenolic contents, and reducing power assay of methanolic and ethanolic extracts of selected lichens from Deosai National Park, Pakistan. Phytochemical analysis of these lichens was also determined in detail. Lichens *Dimlaena oreina*, *Rhizocarpon geographicum*, *Caloplaca marina*, and *Rhizoplaca chrysoleuca* were used for the study purpose. Lichen samples were collected from the Himalayas of Azad Jammu and Kashmir, Pakistan. *Rhizoplacachrysoleuca* showed the highest phenolic contents of 17.19± 0.0386 and 17.55± 0.156 with methanol and ethanol respectively. *Dimlaena oreina* showed the highest reducing power of 2.545 and 2.989, respectively with methanolic and ethanolic extract @125µg/ml as compared to other species. The maximum phytochemical constituents were recorded from *Caloplaca marina.* The present work gives insight into a further investigation for exploring the potential to be used in medicine as natural products.

***Index Terms*- Alpine region; Bioactive contents; Flavonoids; Fungus.**

I. INTRODUCTION

The term lichen denotes an organism that is actually a symbiosis of two simpler creatures [1]. The symbiotic relationship between a fungus and a photoautotrophic organism, by which a fungus gets carbon, and a photoautotrophic organism which may be algae or cyanobacteria gets shelter from fungus is called lichens [2]. Composite organisms, lichens consist of a mycobiont and a phycobiont. Mycobiont is a fungus of class ascomycetes mostly and phycobiont may be algae, or cyanobacteria [3]. Moist habitat is almost occupied by the fungus as this kingdom prefers to grow there. Lichens contain two different kingdoms, but they are suitable to fulfill the requirements of each other even in harsh conditions [4].

Lichens forming fungus and their photoautotrophic partner are the best example of mutualistic relationships [5,6]. Lichens forming fungus is about 85 percent in mutual relation with green algae, 10 percent in mutual relation with cyanobacteria providing carbon and nitrogen to fungus, and the remaining 4 percent with both of them [7, 8]. Approximately 20 percent of all fungus is lichenized [9]. 99 percent of ascomycetes fungi are lichenized and the remaining one percent of class basidiomycetes especially Agaricus species are lichenized [10, 11].

Lichens come in many forms, colors, and shapes [12]. The properties are sometimes plants like but lichens are not plants [13]. Lichens may be tiny leaflets branches called fruticose lichens, flat leaf-like structures called foliose lichens, or flakes that lie on the surface like peeling paint called crustose or other growth forms [14,15]. Lichens have strong secondary metabolites such as phenolic compounds, usnic acid, and depsidones [16, 17]. Lichens have strong resistance against many diseases and are used to cure the infection of viral attacks, have strong resistance against the attack of bacteria or fungi, and against tumor and plant diseases [18,19]. Tannins, alkaloids, saponins, proteins, carbohydrates, glycosides, triterpenoids, and flavonoids were determined in lichens [20, 21]. Lichen *Everniastrum cirrhatum* was used to determine the phytochemical assays [22]. Alkaloids, saponins, triterpenoids, atranorins were found to present in lichen and these substances could be used as a natural source of these metabolites [23,24]. This study aimed to investigate phytochemical screening, phenolic contents, and reducing power assay of methanolic and ethanolic extracts of selected lichens from Deosai National Park, Pakistan.

II. MATERIAL AND METHODS

Site description

Deosai is an alpine region of Pakistan. In 1933 it was declared as National Park due to its ecology. The ecosystem of Deosai much resembles with Tundra due to extreme climatic condition, meadows, pastures and unique natural beauty [25]. Deosai National Park (35˚ 56' N and 71˚ 40' E) is a 1500 km2 alpine plateau located in the western massif of Himalayas, east of Nanga Parbat Peak in close proximity Central Karakoram mountains in Gilgit-Baltistan with an altitudinal range of 3500 to 5200 m. More than half of the plateau

is situated between 4000 to 4500 meter with average daily temperature ranging from – 20°C (January-February) to 12°C (July-August) [26, 27]. Annual precipitation varies from 350 to 550 mm-mostly received during winter as snow due to western disturbances (Fig. 1)

Fig.1 Map of study area.

Collection of lichen samples

Lichen samples were collected from the Himalayas of Azad Jammu and Kashmir, Pakistan. The collection site Deosai is an alpine region of Pakistan. It was declared as National Park in 1993 due to its ecology. The ecosystem of Deosai much resembles with Tundra due to extreme climatic conditions, meadows, pastures and unique natural beauty. The voucher specimens were deposited in the Herbarium, Department of Botany, University of Azad Jammu and Kashmir, Muzaffarabad bearing accession number BJUAJK, 285, BHUAJK, 286, BHUAJK 287 and BHUAJK 288.

Identification of lichen samples

The lichens were kept in air for dry at 26 ^{0}C for one week after cleaning it to remove any superfluous material. The collected species of lichen were identified based on their morphological and anatomical structure by the procedure used by [28].

Preparation of Samples for Analysis

Corona blender was used to ground the dried lichens into fine powder. The specimens were preserved in sterilized bottles at room temperature in the laboratory of Department of Botany, university of Azad Jammu and Kashmir, Muzaffarabad. Samples were stored in clean and dry bottles for further use.

Preparing Methanolic and Ethanolic Extracts

Methanolic extracts of lichens were prepared by the method used by [18]. In conical flask 3g of powder was mixed with 30ml of methanol and in ethanol separately. The mixcher were placed in shaker at room temperature for 3 days. After that it was filtered and kept at room temperature for avaporation of liquid. Crude extracts were stored and kept at 4°C.

Determination of Total Phenolics

Total phenolic compounds in the methanol and ethanol extract were determined according to the method of [29]. 1 ml of the extract (1 mg/ml) in a volumetric flask was diluted with distilled water (46 ml). One milliliter of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 ml of sodium carbonate two percent was added and then allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm in spectrophotometer

Reducing Power

The reducing power of sample was determined according to themethod of [30]. One milliliter of test samples (1000, 500, 250, 125 and 62.5 µg/ml) was mixed with 2.5 ml of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, one percent). The mixtures were incubated at 50 °C for 20 min. Then, trichloroacetic acid (ten percent, 2.5 ml) was added to the mixture and the samples were centrifuged. Finally, the upper layer was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml; point one percent). The absorbance of the solution was measured at 700 nm in spectrophotometer. Higher absorbance of the reaction mixture indicated that the reducing power increased. Ascorbic acid was used as a positive control.

Qualitative Phytochemical Analysis
The phytochemical analysis of crustose lichen was determined by the procedure used by [31,32]

Test for Tannin
Crude extract of 2 ml was mixed with a few drops of five percent ferric chloride solution. Formation of blue color indicated the presence of tannins.

Test for Saponins
Crude extract of 2 ml was mixed with 5 ml of distilled water in a test tube and mixed gently. The formation of transparent form showed the presence of saponins.

Test for Flavonoids
Crude extract of 2 ml was added to 2 ml of ten percent NaOH solution. Yellow to orange color indicated the presence of flavonoids.

Test for Triterpenoids
Crude extract of 2 ml was shaken with 1 ml of chloroform and a few drops of concentrated sulphuric acid. A red brown color formed that is an indication of triterpenoids [33].

Test for Alkaloids
Crude extract 2 ml was added to one percent HCl, steam for 10 minutes. To this, 6 drops of Dragondroff's reagent was added. Reddish brown precipitate will be indicated the presence of alkaloids [34].

Test for Glycosides
Crude extract 2 ml was mixed with 2ml of glacial acetic acid containing 1-2 drops of two percent solution of $FeCl_3$. The mixture was then poured into another test tube containing 2 ml of concentrated H_2SO_4. A brown ring at the interphase indicated the presence of glycosides [31].

Test for Proteins
Crude extract 2 ml was added to 2 ml of HNO_3, boiled in a water bath. Orange color fumes were indicated the presence of protein [31].

Test for Steroids
Crude extract 2 ml of was added to 2 ml acetic anhydride and a few drops of conc. $H_2SO_4$was added. Formation green color indicated the presence of steroids [31].

Statistical Analysis
All research was accomplished in triplicate. The data was documented as mean ± standard deviation and analysed by using statistical package for social sciences (SPSS version 17)

III. RESULTS AND DISCUSSION

Identification of Lichens

The basis for identification of lichens followed on their ecology, morphological and anatomical studies. These were *Dimlaena oreina*, *Rhizocarpon geographicum*, *Caloplaca marina* and *Rhizoplaca chrysoleuca*. These morphological characters are given below*:*

Species Description

***Dimlaenaoreina* (Ach.) Norman**

Dimlaenaoreina (Fig. 2a) is a member of Ascomycota and Caliaciaceae. The lichen is called golden moonglow too. This is a greenish yellow placoid lichen. It has a brown thallus on acid rocks. The lichen grows on an elevation of 400- 2800 meter. Presence of usnic acid in the thallus gives this lichen a yellow appearance. Thallus thin to thick, placodioid, with radiate-plicate marginal lobes, areolate towards the center. It has a worldwide distribution outside the tropics, Australasia and Antarctica.

***Rhizocarpongeographicum* (L.) DC.**

Rhizocarpon geographicum (Fig. 2b) is a member of division Ascomycota and Rhizocarpaceae family that grows on less polluted areas on high altitudes. Each patch of lichen is greenish and lined by black spores. These patches grow adjacent to each other, leading to the appearance of a map or a patchwork field. When circular, or roughly circular, the diameter of this lichen species has been widely used to help determining the relative age of deposits e.g. moraine systems, thus revealing evidence of glacial advances. This lichen species is broadly distributed found in most cold areas with exposed rock surfaces.

***Caloplaca marina* (Wedd.) Zahlbr**

Caloplaca marina (Fig. 2c) is a member of division Ascomycotae and Teloschistaceae family. The lichen is found on calcarious rocks on high altitudes and is a pollution indicator. The thallus of the lichen is orange or orange red in colour that may be continous or fragmented. When fragmented the thallus looks lumpy under a hand lens, when continuous it is areolate and the margins may be ill-defined with almost no lobes. It is never powdery or pruinose. Apothecia are small and either scattered through the thallus or grouped in small clusters, diameters rarely greater than 0.8 mm. The orange disc (a deeper colour than the thallus) surface is concave initially but matures to convex, the apothecium rim also narrows giving the effect that the convex disc is spilling over it.

***Rhizoplacachrysoleuca* (Sm.) Zopf**

Rhizoplaca chrysoleuca (Fig. 2d) is a member of Ascomycota and Lecanoraceae family Upper surface is pale yellow, grey to white and yellow to grey. Apothecia are often crowded. Members of the genus are commonly called rimmed navel lichens because of their umbillicate growth form and lecanorine (rimmed with thallus-like tissue) apothecia, also rock-posy lichen and rockbright.

a. *Dimaena oreina* (Ach.) Norman

b. *Rhizocarpon geographicum* (L.) DC.

c. *Caloplaca marina* (Wedd.) Zahlbr

d. *Rhizoplcachrysoleuca* (Sm.) Zopf

Fig. 2. Selected lichens of Himalayas, Pakistan.

Determination of Phenolic contents

Total phenolic compounds in the methanol and ethanol extracts were determined according to the method of [35]. 1 ml of the extract (1 mg/ml) in a volumetric flask was diluted with distilled water (46 ml). One milliliter of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 ml of sodium carbonate two percent was added and then allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm in spectrophotometer. *Rhizoplaca chrysoleuca* showed high phenolic contents with ethanol (17.55±0.549) and with methanol, phenolic contents (17.19±0.0386). *Dimlaena oreina* showed phenolic contents with ethanol (16.17±0.011) and with methanol (16.91). *Dimlaena oreina* showed high contents with methanol as compared to ethanol. *Caloplaca marina* showed (15.88±0.549) phenolic contents with ethanol while (15.40±0.214) with methanol. Hence, *Caloplaca marina* showed better phenolic contents with ethanolic extract. *Rhizocarpon geographicum* showed (15.32±0.0026) phenolic contents with ethanol while (15.32±0.0026) with methanol. The lichen showed same contents of phenol with ethanol and with methanol. Strong antioxidant activity of lichens correlated with high phenolic contents present in them. Higher content of phenols exerted a stronger radical scavenging effect, suggesting that phenolics are the main agents responsible for their antioxidant activity. Phenolic activity of tested lichens is given in (Table 1).

Reducing power

The reducing power of sample was determined according to themethod of [36]. One milliliter of test samples (1000, 500, 250, 125 and 62.5 µg/ml) was mixed with 2.5 ml of phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferric cyanide (2.5 ml, one percent). The mixtures were incubated at 50 °C for 20 min. Then, trichloroacetic acid (ten percent, 2.5 ml) was added to the mixture and the samples were centrifuged. Finally, the upper layer was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml; point one percent). The absorbance of the solution was measured at 700 nm in spectrophotometer. Higher absorbance of the reaction mixture indicated that the reducing power increased. Ascorbic acid was used as a positive control. *Dimlaena oreina* and *Caloplaca marina* showed the less reducing power as compared to *Rhizocarpon geographicum* and *Rhizoplaca chrysoleuca*. Ascorbic acid used as a positive control and showed the strongest reducing power. *R. geographicum* showed 1.52, 1.619, 1.23, 1.069, 1.199, 1.041, 0.134, 0.179 at 125, 250, 500 and 1000µg/ml respectively at 700nm. *Dimlaena oreina* showed reducing power as 2.545, 2.989, 2.491, 2.917, 1.219, 1.540, 1.540, 0.4029, 0.508 at 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml respectively at 700nm. *Caloplaca marina* showed reducing power as 2.201, 2.377, 1.896, 2.171, 1.453, 1.179, 0.3441, 0.118 at 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml respectively. *Rhizoplaca chrysoleuca* showed 1.679, 1.639, 1.519, 1,539, 0.830, 0.910, 0.251, 0.109 at 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml at 700nm respectively. Ascorbic acid showed less reducing power as compared to samples. Ascorbic acid showed 0.091, 0.019, 0.009, 0.0023, at 700nm. Reducing power of four lichens with their methanolic and ethanolic extracts are given in (Table 2).

Table . 1 Phenolic Activity of selected lichens extracts (mean± SD).

Lichen species	Phenolic contents µg PE/mg extracts	
	Methanol	Ethanol
Caloplaca marina	15.40± 0.214	15.88± 0.549
Dimlaena oreina	16.91± 0.0242	16.17± 0.011
Rhizoplaca chrysoleuca	17.19± 0.0386	17.55± 0.156
Rhizocarpon geographicum	15.32± 0.0026	15.32± 0.0026

Table .2. Reducing power of selected lichens on 700 nm.

Lichen sample	**Absorbance 700nm**							
	125ug/ml		**250ug/ml**		**500ug/ml**		**1000ug/ml**	
	MeOH	**ETOH**	**MeOH**	**ETOH**	**MeOH**	**ETOH**	**MeOH**	**ETOH**
Dimlaenaoreina	2.545	2.989	2.491	2.917	1.219	1.540	0.4029	0.508
Rhizocarpon geographic	1.52	1.619	1.23	1.069	1.199	1.041	0.134	0.179
Rhizoplacachrysoleuca	1.679	1.639	1.519	1.539	0.830	0.910	0.251	0.109
Caloplaca marina	2.201	2.377	1.896	2.171	1.453	1.179	0.3441	0.118
Ascorbic acid	0.036		0.019		0.09		0.0023	

Qualitative Determination of Bioactive Compounds

The bioactive contents analysis of four lichens viz., *Caloplaca marina*, *Dimlaena oreina*, *Rhizoplca chrysoleuca* and *Rhizocarpon geographicum* was conducted for alkaloids, flavonoids, tannins, saponins, proteins, triterpenoids and steroids. The qualitative results of bioactive contents of lichens are given in Table 3.The results showed that all the tested lichens indicate the presence of blue and green colour that designates the presence of tannins (Fig. 2; Table 3). The formation of transparent form indicated the presence of saponins in all extracts (Fig. 2, Table 3). The presence of yellow to orange colour in all extracts indicated the presence of flavonoids (Fig. 2, Table 3). Protein was not seen in the extracts of *R. geographicum* and *R. chrysoleuca* but rest have present (Fig. 2, Table 3). Triterpenoids were not present in *D. oreina* and *R. chrysoleuca*.

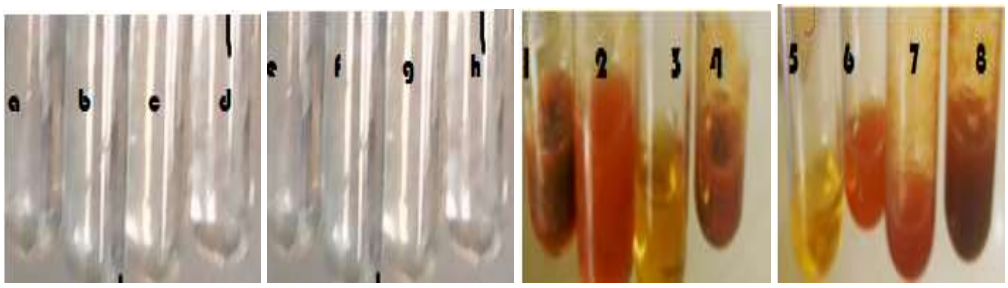
Table 3. Phytochemical constituents of lichens with different solvent extracts.

Lichens	Protein		Gly.		Alk.		Triter.		Flav.		Sap.		Tann.		Ster	
	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E
Rhizocarpon geographi	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

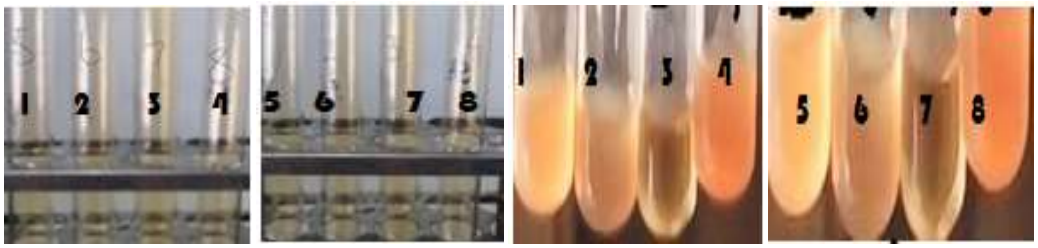
Lichens	Protein		Gly.		Alk.		Triter.		Flav.		Sap.		Tann.		Ster	
	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M	E
Rhizoplacachrysoleuca	-	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Dimlaenaoreina	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Caloplacamarina	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Legends: Gly.= Glycosides; Alk.=Alkaloids; Triter =Triterpenoids; Flav.=Flavonoids; Sap.=Saponins; Tann.=Tannins; Ster.= Steroids

Tannins Flavonoids Glycosoids



Saponins Triterpenoids



Alkaloids Proteins

Steroids

Fig. 2. Qualitative assay for different phytochemicals proteins, tannins, flavonoids, glycosides, saponnins, triterpenoids, alkaloids and steroids of selected lichens.

IV. DISCUSSION

The current study examined reducing power, phenolic contents and bioactive constituents of methanolic and ethanolic extracts of selected lichens such as *Dimlaena oreina, Caloplca marina, Rhizocarpon geomarphicum* and *Rhizoplaca chrysoleuca* from Deosai, Pakistan Himalayas. Methanolic extracts showed strong radical scavengers as compared to ethanolic extracts. These lichens have proton-donating ability and could serve as natural antioxidants. Present research conveyed a number of phenolic compounds flavonoids, tannins, alkaloids and saponins with diverse magnitude in each lichen extract due to which they disclosed such altered scavenging patterns. Reducing power of the extracts was increased with the increasing concentration of the extracts. Ascorbic acid was used as standard. Methanolic extracts showed strong reducing properties then ethanolic one Radical scavenging effect is due to the presence of phenols in lichens. The similar results were reported by Plaza et al. [38] when they analyzed the lichen extracts. Present research conveyed a number of phenolic compounds flavonoids, tannins, alkaloids and saponinis with diverse magnitude in each lichen extract due to which they disclosed such altered scavenging patterns and reducing properties.

All lichens used in present study showed excellent presence of bioactive compounds and radical scavenging potential. Present study deals with the presence of tannins, saponins, proteins, flavonoids, alkaloids, triterpenoids, glycosides and steroids. Preliminary phytochemical analysis of *Dimlaena oreina* and *Caloplaca marina* showed all of the tested phytochemicals. Previously, a study carried out by Aoussar et al. [39] also showed the presence of phytochemicals in these lichens. Glycosides, alkaloids, flavonoids, tannins, and saponins were present in *Rhizocarpon geographicum* and *Rhizoplaca chrysoleum*. Same results were reported by Miral et al. [40].

The qualitative results showed all tested lichens indicate the presence of tannins, saponins and flavonoids in all extracts. Protein was not seen in the extracts of *R. geographicum* and *R. chrysoleuca*, while in rest of lichens that was present. Triterpenoids were not present in *D. oreina* and *R. chrysoleuca*. The same results were reported by Rashmi & Rajkumar [34] when they analyzed the lichen extracts.

V. CONCLUSION

The study concluded the studied lichens possess good source of phytochemicals. The study has explored a new way in the investigation of the use of these lichens in human lives as a natural product. All tested lichens used in the present study showed the presence of phytochemicals and strong reducing power. *Rhizoplaca chrysoleuca* showed the highest phenolic contents and *Dimlaena oreina* showed the highest reducing power as compared to other species. The present study exhibited the bio-pharmaceutical potential of lichens and augments the future investigation for exploring the potential to be used in medicine as natural products.

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