Antioxidant potential of various fractions of lichen species of Gilgit-Baltistan, District Hunza-Nagar

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Abstract

Lichen species Caloplaca trachyphlla and Xanthoparmelia scabrosa were investigated for their antioxidant potential. The methanol extracts and various fractions of these plant species exhibited antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and superoxide inhibitory activity. The radical scavenging activities of the crude extracts and various fractions were significant and more potent than the standard in some cases, as demonstrated by the IC₅₀ values obtained. The medium polar fractions from both C. trachyphlla and X. scabrosa were found to be more potent than least polar and most polar fractions, these findings suggest that lichen from the Gilgit region exhibit significant and promising antioxidant properties. During this study usnic acid is also isolated for the first time from C. trachyphylla.

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Introduction
Lichens are formed by symbiotic associations of fungi and algae and cyanobacteria that results a stable and definite structure (Otalora et al., 2010). The chemical composition of most lichen substances includes phenolics, dibenzofurans, depsides, depsidones, depsones, lactones, quinones and pulvinic acid derivatives (Cetin, et al., 2012; Gaya et al., 2011; Karthikari et al., 2011; Nedeljko et al., 2010; Vertika et al., 2010). They have a diverse chemistry and have been used in traditional medicines and the pharmaceutical and perfume industries. They are known to possess potent antioxidant activity, and therefore they are also used in cosmetics and agriculture (Yilmaz et al., 2004).

Their antioxidant activity was measured by using the DPPH radical scavenging assay and superoxide anion. The lichen compounds are also known to possess antimicrobial and cytotoxic activities (Nigel et al., 1999). Lichens are found to contain different kinds of secondary lichen substances with highly antioxidant activity, and these substances are capable of scavenging toxic free radicals due to phenolic groups present in them (Nedeljko et al., 2011).

The in vitro antioxidant activities of crude extracts and various fractions of C.trachyphlla and X.scabrosa were determined through bioassay guided isolation by exploiting superoxide anion and DPPH radical scavenging assays.

Mammals constantly form reactive oxygen species (ROS) due to oxidative and reductive processes in mitochondria. These arise from oxygen (O₂) derived from respiration, immune responses to foreign antigens, and external exposure to radiation and various chemicals (Valko et al., 2007). However, the antioxidant enzyme systems of the body, including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as various low molecular weight antioxidant such as vitamin C, vitamin E, uric acid and bilirubin, constantly remove the ROS that are formed (Hoda, 2011; Bokov et al., 2004; Asad et al., 2001). Therefore, a balance between the generation of ROS and their removal is obtained and cellular functions are maintained. However, under pathological conditions the antioxidants and ROS balance impairs due to over production of ROS, low antioxidant level or failure to repair oxidative damage, eventually leads to a state called oxidative stress (O'Donovan and Fernandes, 2004). Oxidative stress increases in many clinical disorders, and numerous reports concern the alleviation of these disorders by the administration of antioxidants (Digiesi et al., 2001; Gracy et al., 1999). For instance, diabetic patients have much higher levels of oxidative stress than normal subjects, and some complications of diabetes are relieved by antioxidant treatment (Johansen et al., 2005).

A number of characteristic and unique secondary metabolites are featured to lichens. Out of 800 known secondary metabolites, 80% are associated with the lichenised state. Lichens are exploited in various traditional, homeopathic and naturopathic medicines (Vinitha et al., 2011). Although a number of biological activities of lichens and their metabolites are reported, like antimycobacterial (Muller, 2001), antibiotic (Boustie and Grube, 2005), antiviral (Neamati et al., 1997), analgesic and antipyretic properties (Okuyama et al., 1995), but are yet to be exploited for their full therapeutic potential. The present study was aimed to isolate bioactive constituents of C.trachyphlla and X.scabrosa and to identify the fractions with antioxidant potential found in Gilgit-Baltistan region.

Material and methods
Materials
The lichens were collected from the Challat region of district Hunza-Nagar, Gilgit-Baltistan from an altitude of 4100-4300 m. above sea level. They were identified on the basis of their morphological, anatomical and chemical studies (color reaction and thin layer chromatography). The materials were confirmed as C. trachyphlla and X. scabrosa and specimens were kept for reference at the Karakoram International University. The dry plant material (315 and 398 g respectively) were washed several times with distilled water and extracted with methanol for 2
days. The methanol-soluble fractions of each plant were filtered and the supernatant was concentrated by rotary evaporation. Freeze-drying of the concentrates of *C. trachyphylla* and *X. scabrosa* yielded a greenish (14 g) and reddish (19 g) paste, respectively. These crude extract of *C. trachyphylla* (Gc) was further subjected to liquid column chromatography (CC) on silica gel by using solvent system of n-hexane with the gradient of CHCl₃ (0 to 100%) to get 18 fractions (G1-G18) and chloroform with the gradient increase of MeOH (0 to 50%) to obtain 13 fractions (G19-G31). These crude extract of *X. scabrosa* was further subjected to CC on silica gel by using n-hexane with the gradient of CHCl₃ (0 to 100%) to get 34 fractions (R1-R34) and chloroform with the gradient increase of MeOH (0 to 50%) to obtain 17 fractions (R35-R51). Each of the crude extract and subsequent fractions were evaluated in DPPH and superoxide radical scavenging bioassays. The fraction gr-7 (300 mg) from ethyl acetate extract of *C. trachyphylla* which was eluted by using 7% chloroform in hexane on silica gel was analyzed on TLC for further purification. The TLC showed two spots when developed by using 2% chloroform in hexane. This fraction was then further subjected to column chromatography (silica gel, 200-400 μm mesh) and eluted with gradients of n-hexane: acetone (19:1) and n-hexane: ethyl acetate (9:1, 8:2, 7:3) and finally washed with ethyl acetate (100%). All fractions were then analyzed on TLC and combined the similar fractions. A total of twenty fractions (gr-1 to gr-20) were collected. On further repetitive column chromatography of fraction gr-9 by using 5-12% ethyl acetate in n-hexane on silica gel compound 1 (65 mg) was obtained as white powder which was later characterized and identified as usnic acid.

EI-MS (70 eV) m/z (%): 345 (46), 344 (91), 329 (9), 260 (94), 234 (54), 233 (100), 217 (54), 215 (34)

**In Vitro** DPPH Radical Scavenging Assay

Spectrophotometric method was employed with certain modifications, as described by Donata in 2002 (Bahman *et al.*, 2007). DPPH is a derivative of hydrazine that exists as nitrogen centered radical. The stability of this radical is associated with its conjugated molecular structure. DPPH imparts a deep violet color due to delocalization of the unpaired electron. A decreased in color upon addition of a compound is an indication of the hydrogen donating ability of a test compound. Once the unpaired electron of DPPH becomes paired, the color changes from violet to light green, which is characteristic of hydrazine and its derivatives.

The method was performed by taking 95 μL of DPPH solution and 5 μL of a sample in a micro titer plate. The DPPH Solution was prepared in ethanol and sample under investigation was dissolved in DMSO. The final well concentration for DPPH was maintained at 300 μM and the sample at 500 µg/mL. The reaction mixture was incubated at 37 °C for 30 minutes, and the final absorbance was recorded on a microplate reader at 515 nm. The radical quenching efficacy of compounds was determined by comparison with a DMSO treated blank (control).

The radical Scavenging Activity (% RSA) of extracts was calculated by comparison with a control. % RSA= 100 – \{(As / Ac) X 100}\ 

As= Absorbance of sample, Ac= Absorbance of control.

**In Vitro** Superoxide Anion Radical Scavenging Assay

This protocol was exploited for the determination of superoxide anion scavenging ability of a test compounds by using NADH/PMS system with certain changes, earlier described by Ferda in 2003 (Candan, 2003).

This method involves the generation of superoxide via the reduction of molecular oxygen through natural reducing equivalent β-nicotinamide adenine dinucleotide (reduced form) (NADH). This reaction is catalyzed by phenazine methosulphate (PMS), while nitro blue tetrazolium (NBT) is used as indicator for the generation of superoxide. Upon reduction of NBT by superoxide, a blue color formazan dye is produced, the change in color is monitored by spectrophotometry at 560 nm. The extent of reduction in color by the addition of a test compound
is the measure of its antioxidant potential (Scott & Irwin, 1984).

The superoxide scavenging assay was carried out in a 96-well micro titer plate. Each reaction well contained 10 μL of extract (500 μg/mL), 40 μL of NADH (100 μM), 40 μL of NBT, 20 μL of PMS and 90 μL of phosphate buffer (100 μM, pH 7.4). A buffer was used for the preparation of reagents, whereas DMSO is used with the test compounds. The experiment was carried out at room temperature and generation of superoxide was monitored by employing a spectrophotometer at 560 nm. A control sample was set in parallel for the comparison with the test sample, since the decrease in absorbance of a test sample signifies antioxidant activity.

The radical Scavenging Activity (% RSA) of extracts was calculated by comparison with a control.

\[
\% \text{RSA} = 100 - \left\{ \frac{\text{As}}{\text{Ac}} \times 100 \right\}
\]

As= Absorbance of sample, Ac= Absorbance of control.

**Statistical Analysis**

The experimental data obtained was expressed as ± standard error of mean (±SEM). Statistical analysis was performed by using one-way analysis of variance (ANOVA).

**Results and discussion**

Usnic acid belongs to dibenzofuran class of organic compounds. They exhibit characteristic MS and 1H-NMR spectral features. In EI-MS of dibenzofuran, certain fragment ions predominate. In this class of compounds the presence of methoxy, hydroxy, acetyl, and methyl groups are very common and this can be supported through the presence of a fragment ion at m/z 329 and 217 (fragment ion 'A' and 'D') which arises as a result of bond fission at C-10 and C-8. The cleavage of C-1/C-12 and C-3/C-4 bond lead to fragment ions 'B' and 'C' which appear at m/z 233 and 234 in the EI-MS spectra of usnic acid, respectively (Fig. 2).

<table>
<thead>
<tr>
<th>Code</th>
<th>DPPH Radical Scavenging</th>
<th>Superoxide Anion Radical Scavenging</th>
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<tbody>
<tr>
<td>Gc</td>
<td>55.79±2.59</td>
<td>61.57±1.06</td>
</tr>
<tr>
<td>R6</td>
<td>43.31±1.60</td>
<td>44.74±9.88</td>
</tr>
<tr>
<td>G3</td>
<td>31.86±2.86</td>
<td>31.82±2.35</td>
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<tr>
<td>G5</td>
<td>21.04±4.59</td>
<td>87.25±2.94</td>
</tr>
<tr>
<td>G11</td>
<td>50.87±0.29</td>
<td>34.68±4.35</td>
</tr>
<tr>
<td>G12</td>
<td>38.13±1.46</td>
<td>71.70±3.10</td>
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<tr>
<td>G14</td>
<td>45.32±0.13</td>
<td>25.17±5.86</td>
</tr>
<tr>
<td>G15</td>
<td>43.34±2.00</td>
<td>3.97±1.63</td>
</tr>
<tr>
<td>G3A</td>
<td>76.90±7.34</td>
<td>91.28±1.85</td>
</tr>
<tr>
<td>G4A</td>
<td>45.84±1.89</td>
<td>71.31±1.08</td>
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<tr>
<td>G4</td>
<td>12.93±10.84</td>
<td>87.10±1.38</td>
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<tr>
<td>G5</td>
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<td>87.24±2.94</td>
</tr>
<tr>
<td>G8</td>
<td>41.90±2.25</td>
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<tr>
<td>G9</td>
<td>20.61±1.36</td>
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<tr>
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<tr>
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<tr>
<td>G7A</td>
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<tr>
<td>G6P</td>
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<tr>
<td>PG</td>
<td>91.03±1.60</td>
<td>90.41±2.70</td>
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</tbody>
</table>

RSA= Radical Scavenging Activity, *SEM = Standard Error of Mean, NA= Not Active

**The values are expressed as mean ± standard error of mean, n=3.**
The $^1$H-NMR spectrum of compound 1 (Table 2) showed three highly downfield triplets at $\delta$ 11.00, 13.28 and 18.50 due to the resonance of proton of hydroxy groups at C-10, C-8 and C-3 respectively. Four singlets each integrated for three protons appeared at $\delta$ 1.73, 2.64, 2.08 and 2.65 were assigned to Me-13, Me-15, Me-16 and Me-18, respectively. Proton resonating downfield as singlet at $\delta$ 5.95, was due to H-4 aromatic proton. The comparison of spectral data of compound 1 matched with the literature values already reported for usnic acid (Konig and Wright, 1999).

Table 2. $^1$H-NMR* chemical shift data of compound 1.

<table>
<thead>
<tr>
<th>Position</th>
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<tr>
<td>13</td>
<td>1.73</td>
</tr>
<tr>
<td>15</td>
<td>2.64</td>
</tr>
<tr>
<td>16</td>
<td>2.08</td>
</tr>
<tr>
<td>18</td>
<td>2.65</td>
</tr>
<tr>
<td>3-OH</td>
<td>18.50</td>
</tr>
<tr>
<td>8-OH</td>
<td>13.28</td>
</tr>
<tr>
<td>10-OH</td>
<td>11.00</td>
</tr>
</tbody>
</table>

*Recorded at 300 MHz in CDCl$_3$.

The scavenging ability of radicals by various fractions of $X$. scabrosa (R) and $C$. trachyphylla was estimated via two different bioassays namely, DPPH radical scavenging assay and superoxide anion scavenging assay.

In Vitro DPPH Radical Scavenging Activity

Radicals are continuously produced in cells as byproducts of oxidative reactions and to defend the body against external agents as in phagocytes. DPPH is a stable free radical and is widely used for the evaluation of radical scavenging potential of antioxidant samples (Hatano, 1995). Graph-1 shows the comparison of radical scavenging abilities of different fractions of lichen species. Methanolic extract of $C$. trachyphylla (Gc) showed potential to scavenge DPPH radicals with activity 55.79% On the other hand $X$. scabrosa (Rc) showed a moderate activity (43.31%). The methanolic extracts of both species were further fractionated by using different polarity systems, R5H was found to be the most active fraction (92.60%) against DPPH radicals which was soluble in a hexane/chloroform solvent system (5:95). This indicates that it has greater potential against radicals than the standard antioxidant compound propyl gallate (91.03%). This fraction was followed by G15 with radical scavenging activity 76.90% as given in the table-1. Among other active fractions of $C$. trachyphylla fractionated as different ratios of chloroform, methanol and acetone include; G11 (50.87%) G14 (45.32%) G4A (43.84%) and G13 (43.33%). These results signify that the activity decreases at very high polarity and at very low polarity, since the active constituents are more probably be present at medium polarity. The fractions of $X$. scabrosa (Rc) also showed encouraging activities against DPPH radicals which include; R20 (65.04%) and R21 (58.87%).

Fig. 1. Antioxidant activity of different fractions of $C$.trachyphylla and $X$.scabrosa. The bars represent radical scavenging activity against DPPH and superoxide anion radical. PG= Propyl gallate.
In Vitro Superoxide Anion Radical Scavenging Activity

In biological oxidative reactions superoxide anion radicals are generated when oxygen is reduced. The superoxide anion is normally formed but its effects can be amplified as it converts into other kinds of cell-damaging derivatives and oxidizing agents (Liu & Ng, 2000). The radical scavenging activities of different fractions are compared with standard antioxidant compound propyl gallate, as shown in the graph-1. Methanolic extracts and sub fractions of both the species were also evaluated for their superoxide anion radical scavenging activity. The methanol soluble fractions of *X. scabrosa* (R) and *C. trachyphylla* showed moderate activity, 44.74 and 61.57%, respectively. Almost all fractions of *C. trachyphylla* showed great potential against superoxide anion radicals, the most active fractions was extracted in methanol/chloroform (5:95). G6A with radical quenching activity 97.93% followed by G9 (94.03%), G6P (92.63%), G7A (98.98%) and G15 (91.28%). All these fractions showed a higher ability of radical scavenging than the standard antioxidant compound propyl gallate (90.41%) as shown in table-1. Other fractions which were found to be competitive with propyl gallate include; G8 (88.36%), G5 (87.24%) and G4 (87.10%). The activity increases with the polarity to the medium level, possibly due to the hydrophilicity of phenolic constituents present in lichen species. Moreover, the remaining fractions with notable activity were G10 (76.93%), G12 (71.70%), G4A (71.11%) and G6H (66.73%). Among the fractions of *X.scabrosa*, R10 and R5, extracted in methanol/chloroform (8:92), were found to have significant antioxidant potential with radical scavenging ability, 70.48 and 56.26%, respectively. Although these results are promising, the active fractions need to be evaluated in vivo to validate their therapeutic value.

![Fig. 2. Mass fragmentation pattern of usnic acid.](image)

**Fig. 2.** Mass fragmentation pattern of usnic acid.

![Fig. 3. Structure of compound 1 (usnic acid).](image)

**Fig. 3.** Structure of compound 1 (usnic acid).

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References


[http://dx.doi.org/10.1016/S0009-2797(01)00209-5](http://dx.doi.org/10.1016/S0009-2797(01)00209-5)


http://dx.doi.org/10.1016/S1383-5742(99)00027-7

http://dx.doi.org/10.1186/1475-2840-4-5

http://dx.doi.org/10.1007/s002530100684
(http://dx.doi.org/10.1155/2012/452431)

(http://dx.doi.org/10.4067/S07169760201000020004)

(http://dx.doi.org/10.1006/lich.1999.0241)


(http://dx.doi.org/10.1055/s-2006-958027)

(http://dx.doi.org/10.1016/j.ympev.2010.05.013)

(http://dx.doi.org/10.1016/0003-9861(84)90056-0)

(http://dx.doi.org/10.1016/j.biocel.2006.07.001)

(http://dx.doi.org/10.1007/s11101-010-9189-6)

(http://dx.doi.org/10.1080/14786419.2010.529546)