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The *In Vitro* Antioxidant Properties of Chinese Highland Lichens

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The antioxidant properties of 46 lichen species, collected from the highly UV-exposed alpine areas of southwestern China, were evaluated for their potential therapeutic utilization. The anti-linoleic acid peroxidation activity, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity, reducing power, and total phenolic contents were all assessed *in vitro* in the methanol extract of the lichens. A potent reducing power was detected in a number of the lichen extracts, when compared with butylated hydroxyanisole (BHA). In general, it was found that many of the lichens, with antioxidant properties, contained large quantities of phenolic content. Extracts of *Peltigera praetextata* and *Sticta nylanderiana* were found to exhibit the most potent activity in all of the antioxidant tests. In particular, extracts of *S. nylanderiana* displayed a 1.37 times greater anti-linoleic acid peroxidation activity, when compared with the ascorbic acid used as the positive control. *S. nylanderiana* also possessed the strongest free radical scavenging activity amongst all the tested species, with an inhibition rate of 90.4% at concentration of 330 μg/ml. Activity-guided bioautographic TLC and HPLC analyses were used to establish which compounds were responsible for the potent antioxidant activities of the *S. nylanderiana* extract. These analyses revealed lecanoric acid to be primarily responsible for the effective antioxidant properties of *S. nylanderiana*. Overall, these results have indicated that several highland lichens have the potential of being utilized as novel bioresources for naturally occurring antioxidant therapies.

Keywords: Bioautographic TLC, bioresources, Chinese highland lichens, *Sticta nylanderiana*

Antioxidants are compounds that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions. Currently, a variety of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ), are widely used within the food industry. However, restrictions on the use of synthetic antioxidants are being imposed because of their toxicity to the liver and carcinogenicity [4, 20]. Therefore, the development and utilization of more effective and less harmful antioxidants of natural origins are desirable.

Extreme desiccation and irradiation are known to increase the formation of reactive oxygen species in organisms, and lichens are highly resistant to this type of potential damage [9]. Thus, it is hypothesized that highland lichens may contain considerable quantities of antioxidants in order to protect themselves from the damage of oxidative stress caused by severe exposure to ultraviolet (UV) light. The habitat of the tested lichens in this experiment, Yunnan Province, is located in the Yunnan-Guizhou Plateau of southwestern China, and has an average altitude of between 2,000 and 4,000 meters. The diverse and unique lichen floras in this region have attracted many researchers on a systematic basis [6, 8]. Nevertheless, the chemical and therapeutic potential for the utilization of Yunnan lichen species has not been fully explored.

Therefore, this study focused on the screening of a variety of these highland lichens for antioxidant activity, with the aim of evaluating their medicinal value and their potential as an easily accessible source of natural antioxidants, for utilization as a possible food supplement or within the pharmaceutical industry.

Materials and Methods

Collection and Identification of Lichen Samples

Chinese highland lichen specimens were collected from the Sino-Himalayan region of the Yunnan Province in the People', Republic of China (China). They were previously identified and deposited at the Kunming Institute of Botany of the Chinese Academy of

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Science (CAS), in China. All of the specimens were duplicated and sent to the Korean Lichen Research Institute (KoLRI) at Sunchon National University in the Republic of Korea (Korea).

Preparation of the Methanol Extract

Air-dried and fractioned lichen thalli (5 g) were extracted twice, with 100 ml of methanol for 24 h at room temperature, with a shaking attachment. The combined resultant extracts were then filtered using Whatman filter paper No.1 (Whatman Ltd., U.K.) and then concentrated *in vacuo* at 40°C using a rotary evaporator. The residuals were then dissolved with methanol to a 2 mg extract/ml, and stored in a freezer at −20°C until further study.

Ascorbic Acid–Linoleic Acid Assay

The inhibitory effects of the lichen extract on linoleic acid peroxidation were determined according to the thiocyanate method [14] with a few modifications [12]. A solution without extract served as a blank, or negative control, whereas a solution with ascorbic acid, at the same concentrations as the lichen extract, was used as the positive control. Activity was described as the inhibition percentage (I%) and calculated through the following equation:

$$I\% = (1 - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the negative control, and A_{sample} is the absorbance of the tested extracts.

Determination of Reducing Power

The reducing power of the lichen extract was determined according to the method of Oyaizu [15]. Absorbance at 700 nm indicated the reducing power, and BHA served as the positive control.

Determination of Free Radical Scavenging Activity

The free radical scavenging activity of the lichen extract was measured by DPPH using a modified method of Blois [2, 12]. Free radical scavenging activity of all lichen extracts at the concentration of 330 μg/ml was described as the inhibition percentage of DPPH (I%), with the calculation method of I% being identical to that mentioned above.

Determination of Total Phenolic Content (TPC)

The total quantity of soluble phenolic content in the lichen extract was determined with the Folin–Ciocalteu reagent according to the method of Slinkard and Singleton [18], using catechol as the standard. The concentration of TPC was expressed in micrograms of catechol equivalent per milligram of lichen extract.

Bioautographic TLC Assay for the Free Radical Scavenging Activities of *S. nylanderiana* and *R. intermedia* Methanol Extract

In order to identify the compounds responsible for the strong free radical scavenging activity of selected antioxidant-active lichen extracts, bioautographic TLC assays were performed. These bioautographic TLC assays were based on Chaaib's method [3]. Lichen extract (40 μl, 2 mg/ml) was spotted on a TLC plate (silica gel 60; Merck & Co. Inc., U.S.A.) and developed in solvent system C [Toluene:Acetic acid=85:15 (v/v)]. After drying, the plates were sprayed with a DPPH solution (0.4 mg/ml in methanol) and examined 30 min later. The antioxidant samples appeared as yellow-white spots against a purple background. In order to identify the active compounds responsible for the antioxidant properties, another TLC plate was developed in the same solvent system and visualized by spraying with 10% sulfuric acid. Bioautographic TLC plates were used as a reference to locate antioxidant compounds on prep-TLC plates developed in the same solvent. Silica gel, at the active spot area, was collected and dissolved in acetone, and then filtered. The solution was subsequently analyzed by HPLC (LC-10AT, Shimadzu Corp., Japan) under the following conditions: YMC-Pack ODS-A S-5 μm 150×4.6 mm I.D. column; solvent, methanol:H_2O:H_3PO_4 [80:20:1 (v/v)]; 1 ml/min flow rate; photodiode array detector (range 180–700 nm); detecting wavelength, 254 nm for HPLC and 180–400 nm for UV spectrum analyses; temperature, 40°C. The lichen substances were identified by comparing their retention times and UV spectra with the database of the authentic substances in the Laboratory of Advanced Bio-Production Science, at the Akita Prefecture University, in Japan.

Results and Discussion

The anti-linoleic acid peroxidation activity, the free radical scavenging activity, the reducing power, and the TPC of the methanol extract from the lichen thallus are summarized in Table 1. Generally speaking, the lichen extracts exhibited a very significant inhibition on linoleic peroxidation, and more than half (54.3%) of the species tested showed a stronger activity than that of the ascorbic acid (62.4%) used as the positive control. Several lichen extracts also showed high activity for free radical scavenging when compared with the BHA control (90.9%). Unusually high scavenging activity levels, the highest thus far reported for lichen extracts, were found for *S. nylanderiana* and *P. praetextata*.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [13]. Unlike inhibitory activity against linoleic acid peroxidation, and free radical scavenging activity, all the tested lichen species did not exhibit an effective reducing power, when compared with the BHA positive control. Although *S. nylanderiana*, amongst all the lichen tested, showed the highest level of reducing power, this was still only recorded as being 55.6% of the positive control at the same concentrations (Table 1).

Phenolic compounds are known as high-level antioxidants, mainly as a result of their redox properties, hydrogen donators, and single oxygen quenchers [16]. Lichens also produce some phenolic compounds such as depsides, depsidones, and dibenzofurans [22]. It has been previously demonstrated that the antioxidant capacities of lichen extracts are dependent on their phenolic constituents [5]. Therefore, the TPC of the methanol extracts were measured. The TPC levels were found to vary a great deal amongst the species examined, ranging from 2.8 (*Lobaria yunnanensis*) to 177 (*Nephromopsis stracheyi*) μg catechol equivalent/mg lichen extract. It was noted that many lichen extracts exhibiting high levels of TPC also possessed high levels of

Table 1. Antioxidant activity of forty-six species of Chinese highland lichens.

Lichen species	ALPA	RP	FSA	TPC
	I %	A_{700}	I%	μg CE/mg extract
Bryoria confusa	49.6±1.7	0.14±0.01	10.5±2.9	10.6±1.3
Bryoria himalayensis	74.6±0.7	0.24±0.01	29.3±1.3	70.0±1.0
Bryoria lactinea	74.6±0.4	0.21±0.01	23.1±0.5	80.8±1.2
Bryoria poeltii	82.4±0.1	0.24±0.02	28.5±1.7	36.3±1.7
Cetrelia olivetorum	NA	0.15±0.01	9.5±1.2	72.5±1.4
Cetraria islandica	65.4±0.9	0.28±0.01	20.8±0.7	38.9±3.9
Cetraria laevigata	79.8±0.1	0.19±0.01	29.7±1.6	8.9±0.3
Cetrariopsis wallichiana	70.9±0.9	0.15±0.01	21.2±1.0	5.3±1.0
Cladia aggregata	83.6±0.4	0.25±0.01	21.9±1.7	118.2±1.2
Cladonia amaurocraea	80.9±0.3	0.31±0.01	32.1±2.0	57.5±1.8
Cladonia cervicornis	77.1±0.1	0.22±0.01	29.6±0.6	41.1±1.1
Cladonia corymbescens	78.7±0.1	0.19±0.01	17.1±0.5	41.8±2.0
Cladonia macilenta	79.3±0.2	0.25±0.01	19.3±1.0	87.7±1.9
Cladonia rangiferina	78.7±0.1	0.24±0.01	22.9±0.6	77.8±3.1
Cladonia squamosissma	78.3±0.2	0.22±0.01	25.2±2.7	52.2±0.5
Coccocarpia erythroxyli	79.0±0.6	0.28±0.02	21.5±0.9	24.4±0.5
Evernia mesomorpha	80.7±0.1	0.16±0.01	14.2±1.5	126.6±1.1
Everniastrum cirrhatum	77.2±0.2	0.70±0.01	32.4±1.7	83.5±0.1
Everniastrum rhizodendroideum	69.8±0.1	0.85±0.01	34.2±1.3	112.0±1.8
Hypogymnia hypotrypella	80.3±0.3	0.29±0.01	24.8±0.8	88.4±2.3
Hypogymnia taiwanalpina	82.4±0.3	0.38±0.02	40.9±3.8	60.9±0.8
Lethariella cladonioides	41.2±3.4	0.32±0.01	26.9±1.1	55.4±2.5
Lethariella zahlbruckneri	NA	0.26±0.06	18.1±2.8	109.3±1.0
Lobaria isidiophora	NA	0.28±0.02	31.0±1.2	32.1±4.2
Lobaria kurokawae	72.0±0.4	0.31±0.01	37.3±1.4	30.7±0.4
Lobaria orientalis	NA	0.32±0.01	38.3±0.2	68.4±6.7
Lobaria retigera	NA	0.13±0.01	25.3±0.5	9.7±1.1
Lobaria yunnanensis	NA	0.16±0.01	29.2±2.6	2.8±0.2
Menegazzia terebrata	85.3±0.7	0.31±0.01	48.6±2.0	71.8±2.7
Nephromopsis pallescens	NA	0.13±0.01	15.2±0.8	42.2±1.2
Nephromopsis stracheyi	NA	0.18±0.01	16.6±2.1	177.0±3.3
Nephromopsis yunnanensis	13.5±7.0	0.17±0.01	26.4±0.7	51.0±1.5
Oropogon secalonicus	NA	0.14±0.01	17.8±1.1	16.5±1.3
Peltigera canina	84.9±1.3	0.66±0.01	84.9±0.7	72.6±2.5
Peltigera praetextata	**85.4±0.5**	**1.04±0.01**	**87.8±0.2**	**109.3±0.9**
Pilophorus aciculare	7.0±2.2	0.15±0.01	23.2±0.8	40.7±0.5
Ramalina intermedia	78.8±0.5	0.36±0.01	53.2±0.3	151.7±8.5
Ramalina sinensis	NA	0.19±0.01	20.6±2.3	14.7±0.8
Sticta nylanderiana	**85.5±1.2**	**1.48±0.02**	**90.4±0.5**	**156.1±0.7**
Tuckneraria ahtii	40.7±5.5	0.18±0.01	20.4±1.3	8.4±0.6
Tuckneraria pseudocomplicata	54.6±1.7	0.30±0.01	34.5±1.4	50.6±1.3
Umbilicaria indica	15.0±2.1	0.20±0.01	25.4±1.3	146.4±0.9
Umbilicaria sinoccidentalis	29.5±1.8	0.20±0.01	24.7±3.8	114.5±0.8
Umbilicaria thamnodes	NA	0.15±0.01	17.3±0.3	50.9±5.6
Umbilicaria yunnana	NA	0.19±0.01	21.9±1.0	102.1±1.6
Usnea longissima	31.8±1.0	0.19±0.01	25.4±0.9	142.0±1.5
Ascorbic acid (2 mg/ml)[a]	62.4±3.3	-	-	-
BHA (2 mg/ml)[b]	-	2.67±0.1	90.9± 0.5	-

[a]Positive control of the anti-linoleic acid peroxidation assays.
[b]Positive control of the reducing power and free radical scavenging assays.
ALPA: anti-linoleic acid peroxidation activity; RP: reducing power; FSA: free radical scavenging activity; TPC: total phenolic contents; I%: inhibition percentage; A_{700}: absorbance at 700 nm; CE: catechol equivalent; NA: no activity.
The concentration for all of the lichen extracts was 2 mg/ml in ALPA and RP assays, and 330 μg/ml in the FSA assay.
Values expressed are the mean ± SD of three independent measurements.

phenolic content. It is hypothesized that the latter content may play an important role in the strong antioxidant activities of these lichen extracts. However, not all high TPC species, such as *Nephromopsis stracheyi*, displayed high antioxidant activities (Table 1), suggesting that not all phenolics possess the same antioxidant activity, and that some have a more potent activity, whereas others have a moderate, or even weak, antioxidant activity. It is possible that this result may also be due to the phenolic compounds' synergistic or antagonistic interactions with other phenolics, or differing types of components such as carbohydrates and proteins [17].

It is interesting to note that several of the highland lichens tested in this study exhibit strong free radical scavenging activities at relatively low concentrations (μg/ml) in comparison with earlier studies on the antioxidant properties of lichens from different regions of the world [1, 5]. This indicates that lichens growing in harsh climatic conditions (such as UV-exposed mountains) possess considerably more effective antioxidants than those growing in other regions. This may be as a result of the fact that severe UV light exposure sharply increases reactive oxygen species (ROS) formation in lichens, thereby strengthening their antioxidant defense systems so as to protect themselves from the potential damages caused by ROS.

Bioautographic TLC provides a fast and convenient way for the detection and localization of the active compounds in complicated lichen extracts. The assay was conducted with a *S. nylanderiana* extract, which had exhibited the strongest antioxidant activities amongst the tested lichen species. Two spots (spots 1 and 2) were detected by their yellow-white color reactions on bioautographic TLC plates (data not shown). Spot 1 was relatively weak, whereas spot 2 was much stronger, demonstrating that most of the active antioxidant lichen substances existed in spot 2, making it more worthy than spot 1 for further analysis. Therefore, spot 2 was selectively analyzed by HPLC for its chemical identification, and lecanoric acid was hence identified as the main compound in spot 2 [7, 21] (Fig. 1). The potent antioxidant activity of this compound has also been reported in the extracts of the Antarctic lichen, *Umbilicaria antarctica*, and in the extract of *Parmotrema tinctorum,* commonly found in the tropical and temperate regions [11, 12]. In order to make a comparison between the antioxidant activity of pure lecanoric acid and that of the crude extract, the anti-linoleic acid peroxidation activity and the DPPH scavenging activity were also tested for purified lecanoric acid. The inhibition percentages of lecanoric acid (2 mg/ml) in each assay were 51.5% and 35.4%, respectively. The activity of pure lecanoric acid was thus found to be relatively lower than that of the crude methanol extract of *S. nylanderiana* at the same concentrations (Table 1). This is probably due to the synergistic interactions of lecanoric acid with other unknown compounds in the crude extract,

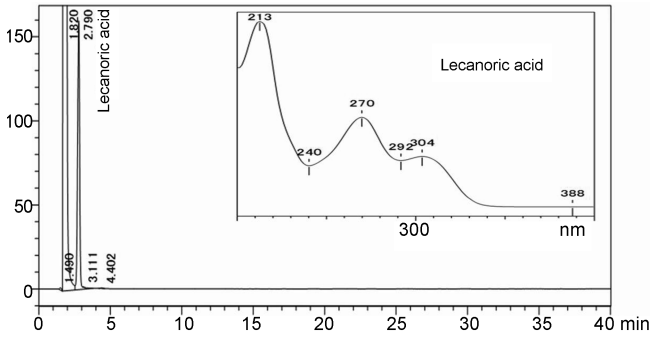

Fig. 1. HPLC chromatogram and UV spectra (insets) of the antioxidant compounds isolated from the extract of *S. nylanderiana.* The HPLC chromatogram and UV spectra of spot 2 show that the second peak is the main component (retention time=2.790 min), and that this substance is identified as lecanoric acid.

which managed to enhance the overall antioxidant activity of the mixture. The identification, and the antioxidant evaluation, of these unknown compounds, along with a probing of the synergistic properties amongst them, is now under way.

Previous studies have suggested that cytoplasmic and membrane-bound antioxidants, such as glutathione, tocopherol, and carotenoids, are essential for the antioxidant activity of lichens [10]. In the present study, we have found that extracellular secondary substances to the lichen also contribute towards the strong antioxidant activity of lichens.

The results obtained in this study are noteworthy, because this is the first study aimed specifically at an evaluation of the bioactivity and therapeutic utilization of Chinese highland lichens. On the basis of the results, it is suggested that the methanol extracts of *S. nylanderiana*, and *P. praetextata* have potent antioxidant activities, and thus exhibit great potential for utilization as bioresources for novel natural antioxidants.

However, this study does not go so far as to suggest the exploitation of the Yunnan highland lichen resources on an industrial scale, owing to their slow rate of growth and relatively low biomass. To apply these natural antioxidant resources in a sustainable way, the effective antioxidants in these lichens may be produced either by fermentation of lichen-forming fungi, or by biosynthesis, such as the heterologous expression of the PKS genes of the lichens in other filamentous fungi [19]. These two latter potential applications are currently being examined for their potential in the mass production of the lichen substances.

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