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Antioxidant and antibacterial activities of lichen *Usnea ghattensis in vitro*

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Abstract

Various solvent extracts of the lichen *Usnea ghattensis* showed good antioxidant activity. A methanol extract prevented lipid peroxidation by 87% followed by 65% in Trolox at 20 μg/ml. It also showed superoxide anion scavenging activity and free radical scavenging activity 56% and 73%, respectively. The known antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and quercetin at similar concentrations showed superoxide anion scavenging activity of 68, 59 and 47% and free radical scavenging activity 83, 77 and 69%, respectively. In addition, these extracts were inhibitory against *Bacillus licheniformis, Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus* with MIC values of 5–10 μg/ml.

Introduction

Recent developments in biomedicals point to the involvement of free radicals in many human diseases. Antioxidants can prevent undesirable oxidation processes by reacting with free radicals, chelating free catalytic metals and also by acting as O_2 scavengers. Restriction in the use of some synthetic antioxidants is being imposed because of their carcinogenicity (Grice 1986, Wichi 1988). Therefore, the development and utilization of more effective natural antioxidants are desired. Further, the spoilage and poisoning of foods by microorganisms are problems that have not yet been brought under adequate control despite the range of robust preservation techniques available. In nature, there are a large number of different types of antimicrobial compounds that play an important role in the natural defense of all kinds of living organisms.

The challenge for today's pharmaceutical industry lies in the discovery and development of new, pharmacological active molecules. In spite of a wide spectrum of biological activities shown by the lichens, they have been long neglected by mycologists and overlooked by pharmaceutical industry because of their slow growth in nature and difficulties in their artificial cultivation (Crittenden & Porter 1991, Yamamoto *et al.* 1998, Behera *et al.* 2003, 2004).

In this report, we describe the antioxidant and antibacterial activities of lichen *Usnea ghattensis in vitro*.

Materials and methods

Lichen culture and extraction

The natural lichen thalli of *Usnea ghattensis* Awasthi were collected from silver oak trees in Mahabaleswar (Satara-District, Maharashtra State) India.

Lichen cultures were started within 7 days after the collection according to the isolation and culture method reported (Yamamoto *et al.* 1985). They were grown at 18 °C with a daily photoperiod (8 h light (400 lux)/16 h dark) for 3 months. Cell mass were then detached from the petri dishes and confirmation of the lichen cultures

were carried out using TLC with benzene/dioxane/acetic acid (180:45:5 by vol.) and hexane/ethyl ether/formic acid (130:80:20 by vol.) (Culberson 1972). Cell mass (14.8 g dry wt) were extracted using 20 ml of either 10% (v/v) acetone, dimethyl sulphoxide (DMSO), methanol or light petroleum (b.p. 40–60 °C) at room temperature. The extracts were then filtered, concentrated 4-fold under vaccum and freeze dried. The maximum permissible concentrations 0.5, 1 or 2 mg of dried extract were then dissolved in 1 ml of acetone, DMSO, methanol or water for the preparation of test stock solutions. This stock test solution further diluted in the corresponding solvent to achieve the test solution containing 5, 10 or 20 μg extract and were used for the determination of their activities. Same procedure is also followed for the test solution of desired concentrations of the known antioxidants used as standards.

In all cases, three independent experiments, each with duplicate measurements were performed. The results shown are the means of these measurements.

Microorganisms

Microorganisms used for screening of antimicrobial activities are given in Table 2. The screening of lichen extracts against bacteria were carried out using disk diffusion and turbidometric methods (European Pharmacopoeia 1971, Aiken 1977).

Antioxidant assay

It is well reported that antioxidants are acting as O_2 scavengers and free radical terminators (Sanchez-Moreno *et al.* 1999). Thus, the scavenging effects of various solvent extracts of the lichen *U. ghattensis* has been carried out in different antioxidant assay systems. The Trolox equivalent antioxidant capacity (TEAC) of various solvent extracts of the lichen *Usnea ghattensis* was measured according to the method of Miller *et al.* (1995). The TEAC assay is based on scavenging of long-lived radical ions, ABTS, by the Trolox (a water-soluble vitamin E analogue, Aldrich). TEAC value is expressed as mM Trolox solution having the antioxidant equivalent to a 0.1% (w/v) extract solution.

The inhibition of lipid peroxidation by the extracts were determined using the method of Liegeois *et al.* (2000). Superoxide anion scavenging activities determined using method of Nishimiki *et al.* (1972). The free radical scavenging activity was determined using method of Blois (1958).

Total phenolic assay

Total phenolics content was determined with Folin-Ciocalteu reagent using pyrocatechol as a standard according to the method (Slinkard & Singleton 1977).

Results and discussion

The antioxidant and antibacterial activities of the various extracts of *Usnea ghattensis* were investigated. The methanol extract had the equivalent antioxidant activity of 4 mM Trolox which was higher than all the other extracts (Table 1). The methanol extract at 20 μg prevented lipid peroxidation by 87%, whereas a similar concentration of Trolox had only 65% inhibition of lipid peroxidation (see Figure 1). There was a significant difference ($p < 0.05$) observed between the inhibition of lipid peroxidation of samples with extract and samples without extract. Further, the methanol extract at 20 μg showed 56% superoxide anion scavenging activity whereas at similar concentration of known antioxidants, butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and quercetin, showed superoxide anion scavenging activity 68, 59 and 47%, respectively. Other extracts had only 7–27% superoxide anion scavenging activity. Additionally, the maximum of 73% free radical scavenged by 20 μg methanol extract was higher than the free radical scavenged (11–43%) by the other extracts also at 20 μg. However, at similar concentrations, butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and quercetin showed free radical scavenging activity 83, 77 and 69%, respectively. No extracts had a higher superoxide anion and free radical scavenging activity than that of BHT. The differences in the antioxidant activities of various solvents might be due to their different capabilities to extract bioactive substances. Although, in general, increasing of the extract concentration (from 5 to 20 μg/ml) and incubation time period (30–90 min) increased the inhibition of lipid peroxidation, superoxide anion and free radical scavenging activities but beyond the

Table 1. Trolox equivalent antioxidant capacity (TEAC) of various lichen extracts solution 0.1% (w/v).

	TEAC (mM)	Inhibition of lipid peroxidation (%)	Free radical scavenged (%)	Superoxide anion scavenged (%)	Total polyphenol (mg/g dry extract)
Extracts					
DMSO	0.6	17	11	7	12
Methanol	4	87	73	56	35
Acetone	2	55	31	18	14
Light petroleum	0.8	38	43	27	9
Standard antioxidants					
Trolox		65			
Butylated hydroxyanisole			77	59	
Butylated hydroxytoluene			83	68	
Quercetin			69	47	

Antioxidant activities; Inhibition of lipid peroxidation, free radical scavenging and superoxide anion scavenging activities of various extracts and the known antioxidants at a concentration of 20 μg/ml.
TEAC assay: $ABTS^{\bullet+}$ radical cation generated by the interaction of $ABTS^{\bullet+}$ (100 μM), H_2O_2 (50 μM), and peroxidase (4.4 unit/ml). 250 μl extract was mixed with an equal volume of $ABTS^{\bullet+}$, H_2O_2, peroxidase, and deionized water. A_{734} was then measured. TEAC value is expressed as mM of Trolox solution having the antioxidant equivalent to a 0.1% (w/v) extract solution. For Inhibition of lipid peroxidation: 30 μl 16 mM linoleic acid was mixed with 2.81 ml 0.05 M phosphate buffer, pH 7.4, at 40 °C. Oxidation was carried out with 20 μl each of extract. A_{234} was then measured. Free radical scavenging effect of the extract measured at A_{517}. Superoxide anion scavenging activities of the extract was measured at A_{560}. Samples containing extract or known antioxidant conc. 20 μg/ml was used in lipid peroxidation assay, superoxide anion scavenging and free radical scavenging activity assay. Trolox, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene and quercetin used as positive control.

extract concentration of 20 μg/ml after 90 min incubation did not increase the antioxidant activities. This suggests that the antioxidant activities are concentration and time-dependent.

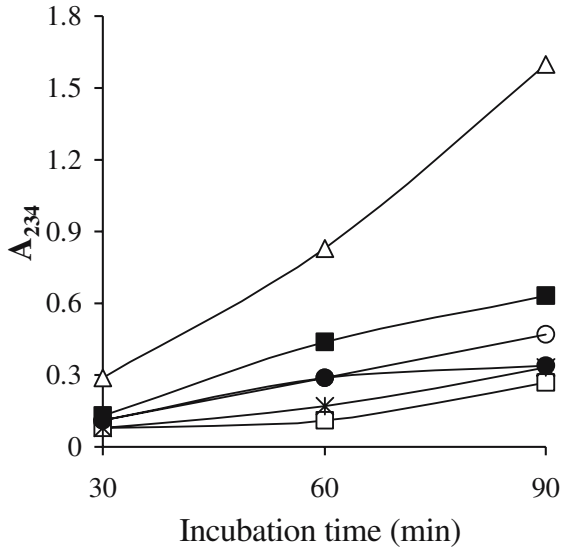

Fig. 1. Inhibition of lipid peroxidation by the *Usnea ghattensis* extract (20 μg/ml) and Trolox (20 μg/ml) measured by conjugated diene hydroperoxides formation at A_{234} with time. Δ = control, ■ = DMSO, □ = Methanol, ○ = Acetone, * = light petroleum, ● = Trolox.

The methanol extract of *U. ghattensis* had a higher phenolic content than the other extracts (Table 1). The correlation ($r^2 = 0.755$, $p < 0.05$) between the total phenols in the extracts and the inhibition of lipid peroxidation by the extracts is shown in Figure 2. This suggests that the phenol content of the extract correlates with the antioxidant activity.

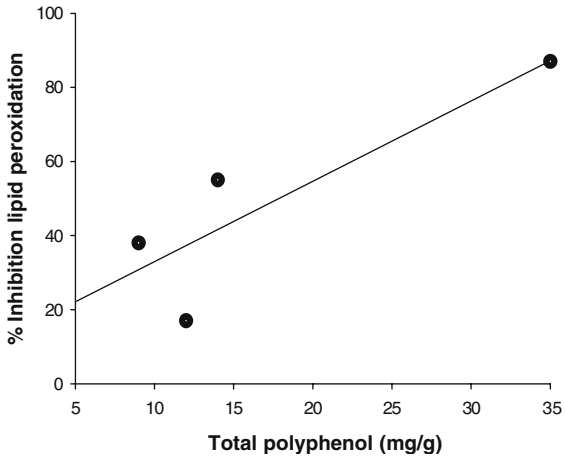

Fig. 2. Correlation between inhibition of lipid peroxidation by the *U. ghattensis* extract and the total polyphenol content in the extract; $y = 2.1698x + 11.278$, $r^2 = 0.755$.

Table 2. Antibacterial activity (zone of inhibition, cm) of *Usnea ghattensis* extract by different solvent and standard antibiotics.

Bacteria	Extract (μg extract)									Ampicillin (10 μg)	Streptomycin 10 μg)
	Methanol			Acetone			Light petroleum				
	5	10	20	5	10	20	5	10	20		
S. aureus	2.0	2.2	2.6	2.0	2.2	3.0	2.5	2.7	3.5	5.0	2.6
B. licheniformis	1.6	1.8	2.5	1.6	2.0	2.5	2.0	2.7	3.0	1.0	2.1
B. subtilis	1.5	2.0	2.3	1.9	2.1	2.4	1.6	1.9	2.1	1.8	2.4
B. megaterium	1.5	1.7	2.2	1.9	2.0	2.4	1.3	1.7	2.0	2.0	2.2

Muller-Hinton agar (25 ml) was inoculated with 100 μl bacterial suspension of 0.6×10^6 c.f.u./ml. Sterile disks (diameter, 6 mm) were impregnated with 10 μl of test solution. Ampicillin and streptomycin were used as positive control. The inoculated plates were incubated for 18–24 h at 37 °C, after which the size of the inhibition zone was measured. *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus* were provided by the Department of Microbiology, MACS collection of microorganisms (MCM) WFCC CODE-561. Agharkar Research Institute, Pune, India.

The acetone, methanol and light petroleum extracts were effective against *B. licheniformis*, *B. megaterium, B. subtilis* and *S. aureus* (Table 2). Furthermore, the size of zone of inhibition increased as the concentration of extract increased suggests that the effect was concentration dependent. The minimum inhibitory concentration (MIC) of the extract was 5–10 μg/ml.

The methanol extract of *U. ghattensis* has high antioxidative and antibacterial potential. Lichens produces a number of secondary metabolites many of which are phenolic compounds. Since, a significant correlation was obtained between the total phenols in the extracts and the inhibition of lipid peroxidation by the extracts (see Table 1 and Figure 2), we believe that the antioxidant activity shown by the extracts probably due to the presence of phenolic compounds. Our results are in agreement with those that reported the ability of phenolic compounds to scavenge free radicals and active oxygen species (Hatano *et al.* 1989, Duh *et al.* 1999) and to show antimicrobial activity (Rauha *et al.* 2000). Hence *U. ghattensis* deserves to be explored for its bioactive molecules. There is however, a need to improve the growth rate of the cultured tissue in the laboratory to harvest large quantities of cells and thus metabolites.

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