Advanced Journal of Microbiology Research Vol. 2008
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Lipid peroxidation and tyrosinase inhibition by lichen symbionts grown *in vitro*

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Accepted 25 January 2008

The symbionts of lichen species *Arthothelium awasthii*, *Heterodermia podocarpa*, and *Parmotrema tinctorum* were isolated and cultured on various nutrient media incubated in a growth chamber. Methanolic extracts of one year old symbiont cultures were taken for evaluating their potential to inhibit lipid peroxidation and tyrosinase enzyme activity. The extracts of all cultured lichen-symbionts have shown a concentration-time dependent inhibition of lipid peroxidation and tyrosinase activities. IC_{50} values for inhibition of lipid peroxidation by culture-extracts were 15.7 µg for *A. awasthii*, 12.68 µg for *H. podocarpa* and 11.47 µg for *P. tinctorum*. Testing the impact of the extracts on tyrosinase activity, we found IC_{50} of 8.71 µg (*A. awasthii*), 14.55 µg (*H. podocarpa*) and 12.44 µg (*P. tinctorum*). According to their IC_{50} the tested extracts turned out to be more effective for the tested parameters than the standard antioxidant Trolox (IC_{50} for lipid peroxidation 16.13 µg) and Kojic acid tyrosinase inhibitor (IC_{50} for tyrosinase inhibition 17.63 µg). The results suggest possible applications of lichen substances in the extracts of *A. awasthii*, *H. podocarpa*, and *P. tinctorum* symbionts as natural tyrosinase inhibitors.

Keyword: Lichen culture, Tyrosinase inhibition

INTRODUCTION

Lichens are symbiotic organisms composed of a fungal partner (mycobiont) in association with one or more photosynthetic partners (photobiont) . The complex symbiosis of the bionts allows to form the lichen thallus which is a separate entity neither resembling the myco-biont nor the photobiont alone. Therefore lichens are referred to as individual organisms but still named after the fungal partner, which is dominating the sym-biosis in most of the cases. Lichens are an integral part of all terrestrial ecosystems and especially successful in some extreme environments like the Antarctica. Lichens are important food source for many animals, and they do also play an important role for humans in some countries where they are part of their diet or used in traditional medicine (Upreti and Chatterjee, 2007) . Lichens and their natural products have a long tradition of being used for decorations, brewing and distilling, perfume, dying Indus-try, food and natural remedies (vide Oksanen, 2006). They also demonstrated to evidence a number of se-condary metabolites, which may protect them against physical stress or biological attacks during their slow growth (Crittenden and Porter, 1991; Herbert, 1992; Kahng et al., 2004). Several studies have recently demonstrated a range of different biological activities of lichen secondary metabolites. Especially substances of the depside- and depsidone- classes have been shown to exhibit antiviral, antibiotic, antitumor, allergenic and antioxidative activities as well as plant growth inhibitory effects (Muller, 2001; Hidalgo et al., 1994; Huneck, 1999; Honda and Vilegas, 1999). With this background information, we have already investigated various naturally grown lichens and some cultured symbionts /mycobionts for their capacity for lipid peroxidation and tyrosinase- inhibition. The results are reported elsewhere (Behera et al., 2002; 2003; 2005 a, b and c; 2006 a, b, c). As compared to other organisms, the biological activities reported are very less in lichens (Miao et al., 2001). The main reason for the lack of experimental data about the activities of lichen substances is the slow growth of lichen thalli in nature (in the range of millimeters to centimeters per year; Hale, 1983, Yoshimura et al., 1993) and restrict-tions in culturing and maintenance of lichen mycobionts under laboratory conditions (Stocker-Worgotter, 1995; Miao et al., 2001). However, in recent past Yamamoto et al. (1985) have developed a culture technique using thal-lus fragments which is an effective and time-saving alter-native to methods based on spores. Employing the frag-

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ment isolation technique allows to establish lichen cultures in much shorter time than before. Producing cultured mycobionts / symbionts in higher quantities in much shorter time greatly facilitated isolation of lichen substances and screening for their biological activities (Higuchi et al., 1993; Yamamoto et al., 1993; 1998).

Tyrosinase or polyphenol oxidase (monophenol, o-diphenol: oxygen oxidoreductase; EC 1.14.18.1) is a copper enzyme that catalyzes two different reactions using molecular oxygen: the hydroxylation of mono-phenols to o- diphenols (monophenolase activity) and the oxidation of the o-diphenols to o-quinones (diphenolase activity; Sanchez-Ferrer et al., 1995), this enzyme is widely distributed in plants, microorganisms and animals where tyrosinase is responsible for melanization. The formation of melanin in the human body influenced or reduced by several mechanisms, including anti-oxidation, direct tyrosinase inhibition, melanin inhibition of migration from cell to cell and hormonal activities etc. (Prota and Thomson, 1976; Pawelek and Korner, 1982). Recently tyrosinase inhibitors have been used frequently in cosmetics and depigmenting agents for hyperpigmentation (Funasaka et al., 2000). Therefore a concerted effort has been made to search for naturally occurring tyrosinase inhibitors from various organisms, many of them being largely free from harmful adverse effects (Sasaki and Yoshizaki, 2002).

The present study reports lipid peroxidation and tyrosinase inhibitory features of one year old cultured symbionts of three lichen species *A. awasthii*, *H. podo-carpa* and *P. tinctorum*.

MATERIALS AND METHODS

Lichen material

Thalli of the lichen species namely *A. awasthii* (crustose; barbatic acid and two unknown substances), *H. podocarpa* (fruticose; atranorin and zeorin), and *P. tinctorum* (foliose; atranorin and lecanoric acid) were collected from different localities in Mahabaleswar (Satara- District, Maharashtra State) India. A part of the material of each species has been preserved as dried herbarium specimen in the Ajrekar Mycological Herbarium (AMH), Pune, India

In-vitro culture of lichens

The lichens *A. awasthii*, *H. podocarpa* and *P. tinctorum* were cultured under laboratory conditions following the method described by Yamamoto et al. (1985). The experimental details of symbionts derived as cell aggregates from various lichen species *in vitro* have been reported in our previous papers (Behera et al., 2000; 2002; 2004; 2005 a, b and c; 2006 a, b, c). The following culture media were used: Malt-Yeast Extract (MYE); Lilly Barnett (LB); Murashige and Skoog (MS); Bold's Basal Medium (BBM); Bischoff and Bold (Vide Ahmadjian, 1993) and Modified Bold's Basal medium (Behera et al., 2000).

After every three months, the development of lichen symbionts of different species was checked by mounting the tissue in cotton blue and confirmed by transmission light microscope.

Identification of lichen substances

The presence of mycobionts and photobionts in culture were confirmed by the presence of lichen substances produced. Culture cell aggregates of lichen *A. awasthii, H. podocarpa* and *P. tinctorum* were extracted in methanol by soxhlet extractor and high-performance liquid chromatographic (HPLC) analysis was carried out on Agilent 1100 system with 360 autosampler, C8 (ZORBAX) column (Eclipse × DB-C8, 4.5 × 150 mm, 5 µm) and UV spectrophotometric detector, at 28^{O}C with methanol-water-phosphoric acid (80:20:0.9, v/v/v). The detection wave length was 254 nm and the injection volume was 5 µl, with a flow rate of 1 ml following Feige and Lumbsch (1993).

Retention time (Rt) and peck symmetry of produce cultured lichen substances were compared with the substances of their respective natural thalli. The retention times were 2.45 min for barbatic acid, 2.21 min for atranorin, 4.45 min for norstictic acid, 2.35 min for zeorin and 7.49 min for lecanoric acid respectively. The retention index values (RI) were also calculated using benzoic acid and the quantitative determination of respective lichen substances were done, where the integrated areas under the particular peaks reflect the concentration of the substances present (Huneck and Yoshimura, 1996).

Electron donating ability and lipid peroxidation inhibition activity

Total extracts of the cultured symbionts of *A. awasthii, H. podocarpa,* and *P. tinctorum* were tested for electron donating ability and lipid peroxidation inhibition activity by using 1,1-diphenyl-2- picryl-hydrazil (DPPH$^{\cdot}$) free radical (Blois, 1958) and inhibition of lenoleic acid peroxidation by Liegeois et al. (2000). Trolox a water soluble vitamin E analogue was used as positive control. The details of the minor modifications to the procedure are reported earlier by us (Behera et al., 2005 a, b, c).

Tyrosinase inhibition assay

Tyrosinase- inhibitory activity of cultured lichens *A. awasthii, H. podocarpa,* and *P. tinctorum* were determined according to Higuchi et al. (1993). Detailed procedures were reported earlier by Behera et al. (2006c). The positive control was done with 1% (w/v) solution of kojic acid as a standard tyrosinase-inhibitor.

Polysaccharide assay

The polysaccharide content of the cultured symbionts of lichen *H. podocarpa, A. awasthii* and *P. tinctorum* were determined using the method described by Dubois et al. (1956).

Determination of total soluble phenolic contents

Total soluble phenolics in the methanolic extract of cultured, *A. awasthii, H. podocarpa* and *P. tinctorum* were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) using pyrocatechol as a standard.

Antioxidant component analysis by thin layer chromatography

Antioxidative component in the total extract of the cultured symbionts from *A. awasthii*, *H. podocarpa*, and *P. tinctorum* were analyzed following the procedure described by Espin et al. (2000). The detail of the procedure with minor modification was described earlier by Behera et al. (2007).

Table 1. Lichen symbiont growth after complete one year under in vitro conditions and their polysaccharide and total phenol content. Data presented are mean of 5 replicates

					Nutrient medium	
	Malt-Yeast	**Bischoff and Bold**	**Lilly-Barnet**	**Murashige and Skoog**	**Bold Basal**	**Modified Bold Basal**
Arthelium Awasthii						
Dry biomass (g)/petriplate	0.57	0.34	1.67	0.27	2.31	5.74
Soluble extract (µg/d.wt.)	17.82	3.42	43.4	7.43	34.78	83.11
Polysaccharide (mg/g)	7.31	2.83	8.81	5.56	7.62	17.36
Total phenol (mg/g)	21.47	16.46	36.2	11.4	19.73	41.22
Heterodermia podocarpa						
Dry biomass (g)/petriplate	0.48	0.77	0.83	0.33	1.07	2.23
Soluble extract (µg/d.wt.)	3.42	6.31	48.7	4.71	23.63	57.54
Polysaccharide (mg/g)	5.74	4.85	7.47	4.54	11.38	21.37
Total phenol (mg/g)	17.8	7.32	14.42	11.62	23.86	33.46
Parmotrema tinctorum						
Dry biomass (g)/petriplate	0.24	0.41	0.78	0.29	1.26	3.16
Soluble extract (µg/d.wt.)	2.76	7.25	38.32	8.84	27.37	44.24
Polysaccharide (mg/g)	6.82	8.56	7.26	4.66	11.33	27.32
Total phenol (mg/g)	12.37	7.68	28.28	7.69	27.48	43.83

Statistical analysis

Experimental results were mean of five replicates. P- values < 0.05 were regarded as significant and P-values < 0.01 very significant.

RESULTS

Lichen growth

Lichen symbionts of *A. awasthii*, *H. podocarpa* and *P. tinctorum* were successfully isolated and cultured on various nutrient media. The growth of the cultured symobionts was monitored and documented in Table.1. The Malt-Yeast Extract (MYE), Bischoff and Bold and Murashige and Skoog (MS) media favoured the growth of mycobiont in three cultures; *A. awasthii*, *H. podocarpa* and *P. tinctorum*. The growth of symbionts in all cultures was found in the Lilly Barnett (LB), Bold's Basal Medium (BBM) and Modified Bold's Basal media after two to three month incubation in the growth chamber. However, their growth rate and production of lichen substance were varied irrespective of the media used. The cultures having symbionts only could be able to produce lichen substances as they were produced in nature. Only LB, BBM and Modified Bold's Basal media were found most suitable for the growth of symbionts and production of lichen substances under laboratory conditions.

Lipid peroxidation inhibition (LPI)

Lipid peroxidation inhibition by different concentrations (2, 5, 10 or 20 µg/ml) of the methanolic extract of cultured symbionts of three lichen species *A. awasthii*, *H. podocarpa* and *P. tinctorum* were presented in Figure 1. The LPI showed by *A. awasthii* ranging from 2.11 to 68.4%; *H. podocarpa* 3.43 to 52.7% and *P. tinctorum* 3.58 to 71.3% whereas Trolox a water soluble vitamin E ana-logue as standard antioxidant from 5.41 to 89.2%. This data indicates a concentration-depending LPI activity of the extract or standard increased. The activity of the extracts was found to be similar to that of Trolox.

In order to know the LPI activity of the extract under different incubation period, we have measured the rate of LPI activity of the extract at 0, 5, 15 and 45 min incubation and the data are presented in Figure 2. Significant increment in LPI by the extract was found when incubation period increased from 5 to 15 min. However, no further addi-tional effect was observed when the incubation period was extended to 45 min. These results indicate that the LPI activity by the extracts is time-dependent.

Electron donating ability (EDA)

The electron donating ability of the lichen extract concentrations 2, 5, 10 or 20 µg in the assay mixture were shown in Figure 3. The extract of cultured symbionts of lichen species *A. awasthii*, *H. podocarpa* and *P. tinctorum* showed EDA 2.56 to 68.4%; 1.58 to 53.7%; 1.13 to 57.2% respectively. Trolox a water soluble vitamin E ana-logue as standard antioxidant showed EDA 3.71 to 78.7%. from 10 to 20 µg/ml in the assay mixture the TI activity significantly increased. TI activity rates of culture extracts

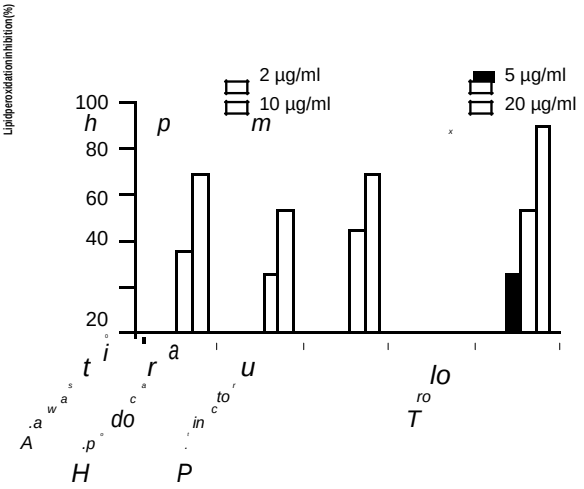

Figure 1. Lipid peroxidation inhibition by the extract of various concentrations of the one year old cultured symbionts of lichen species. Data plotted are mean of five consecutive readings.

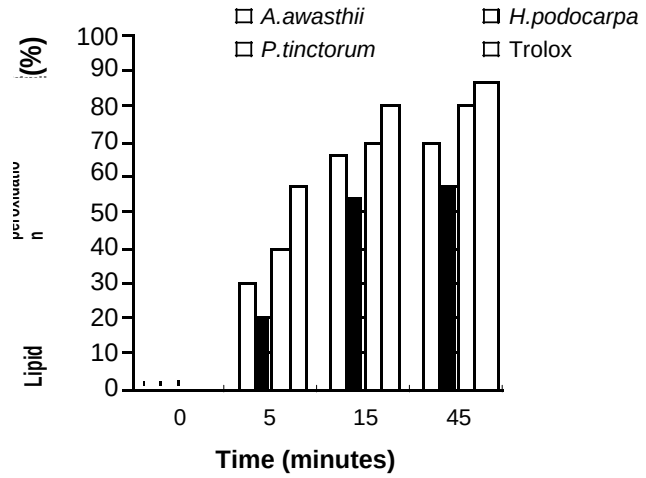

Figure 2. Rate of lipid peroxidation under various time period by the extract (20 µg/ml) of one year old cultured symbionts of lichen species. Trolox a water soluble vitamin E analogue used as commercial antioxidant for comparison. Data plotted are mean of five consecutive readings.

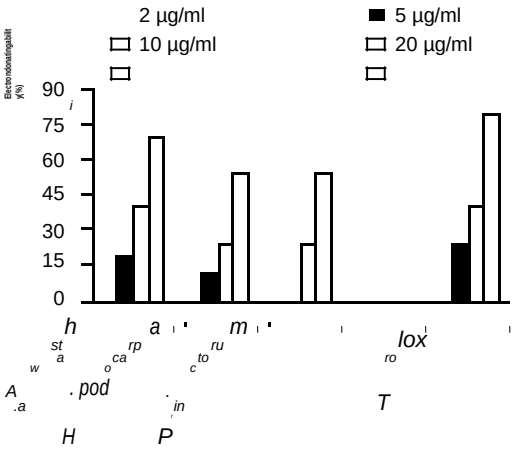

Figure 3. Electron donating ability by the extract of various concentrations of one year old cultured symbionts of lichen species. Trolox a water soluble vitamin E analogue used as commercial antioxidant for comparison. Data plotted are mean of five consecutive readings.

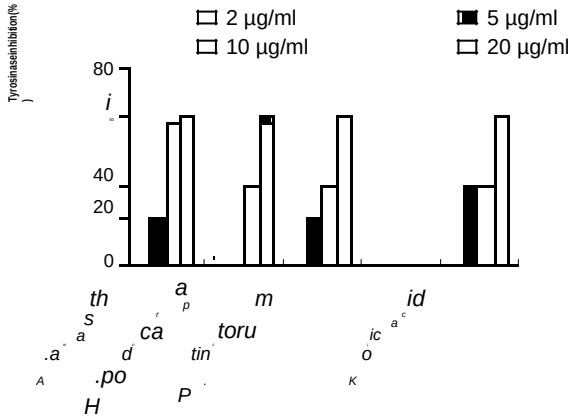

Figure 4. Tyrosinase inhibition by the extract of various concentrations of one year old cultured symbionts of lichen species. Kojic acid used as standard tyrosinase inhibitor for the comparison. Data plotted are mean of five consecutive readings.

Tyrosinase inhibition activity (TI)

Methanolic extracts of cultured symbionts of three lichen species *A. awasthii*, *H. podocarpa,* and *P. tinctorum* were tested in different concentrations (2, 5, 10 or 20 µg/ml) for their tyrosinase inhibition activity presented in Figure 4. The TI-values of *A. awasthii* were ranging from 4.62 to 67.2%; *H. podocarpa* showed values of 1.2 to 57.8% and *P. tinctorum* 7.4 to 63.6%. Kojic acid, which was used for comparison as standard tyrosinase inhibitor showed TI 7.8 to 68.9%. When the extract concentration increased in a time dependent manner are presented in Figure 5. The rate of TI activity increased significantly, when the time of incubation of was increased from 5 to 15 min. These results indicate that the TI activity is dose-time dependent. In general, The TI activity of the culture extracts was resembling with kojic acid.

Polysacharide and soluble phenol content of the cultured symbionts

Polysaccharide and soluble phenol content produced by the cultured symbionts of the lichen species in this study are shown in Table 1. Irrespective of the medium used, the polysaccharide content of *A. awasthii* was ranging from 2.8 to 17.3 mg/g dry weight (d.wt); *H. podocarpa* from 4.5 to 21.3 mg/g d.wt. and *P. tinctorum* from 4.6 to 27.3 mg/g d.wt. Similarly soluble phenol content were found in *A. awasthii* was ranging from 11.4 to 41.2 mg/g; *H. podocarpa* 7.3 to 33.4 mg/g; *P. tinctorum* 7.6 to 43.8 mg/g respectively. In general, as the incubation period increased, the symbionts of three lichen species grown in the media LB, BBM and Modified Bold's Basal media significantly increased the polysaccharide and phenol content.

Table 2. Half-inhibiting concentration (IC_{50}) of the extract of one year old cultured symbionts of lichens for the inhibition of lipid peroxidation (LPI), tyrosinase (TI) and the electron donating ability (EDA). Data presented are the mean of five consecutive readings of the extract in assay.

	Half-inhibiting concentration (IC_{50}, µg/ml)		
	LPI	**EDA**	**TI**
Arthothelium awasthii	15.7 ± 2.7	13.23 ± 1.14	8.71 ± 0.83
Heterodermia podocarpa	12.68 ± 1.32	18.37 ± 2.08	14.55 ± 0.33
Parmotrema tinctorum	11.47 ±0.68	13.64 ± 1.71	12.44 ± 0.17
Trolox (standard antioxidant)	16.13 ± 1.03	14.57 ± 3.26	-
Kojic acid (tyrosinase inhibitor)	-	-	17.63 ± 3.18

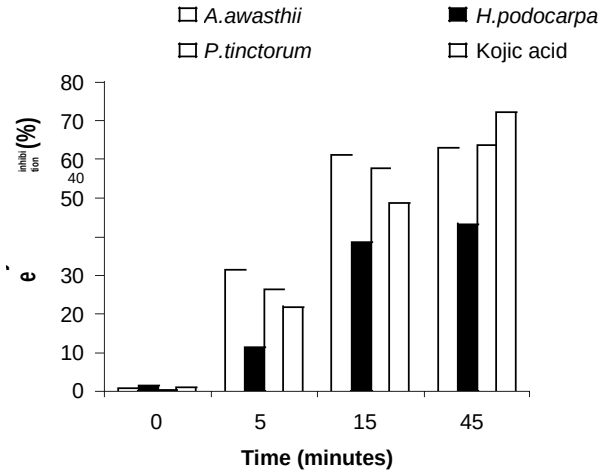

Figure 5. The rate of tyrosinase inhibition under various time period by the extract (20 µg/ml) of one year old cultured symbionts of lichen species. Kojic acid a commercial tyrosinase inhibitor used for comparison. Data plotted are mean of five consecutive readings.

Lipid peroxidation inhibition, electron donating ability and tyrosinase inhibition activities in relation to polysaccharide and polyphenol content

In order to know the significance of the presence of polysaccharide and phenols in the extract on the lipid peroxidation inhibition, electron donating ability and tyrosinase inhibition, we have correlated polysaccharide content with LPI, EDA and TI. The relationship found were $R^2 = 0.484$, $R^2 = 0.536$, $R^2 = 0.762$ respectively. Further correlation between phenol content and LPI, EDA and TI the relationship found were $R^2 = 0.831$, $R^2 = 0.693$, $R^2 = 0.816$ respectively.

Calculation of 50% Inhibition concentration (IC_{50})

IC_{50} value of the lichen extracts for the lipid peroxidation inhibition (LPI), electron donating ability (EDA) and tyrosinase inhibition (TI) and the commercial standards were calculated by extrapolation from concentration/effect regression lines obtained from 3 to 4 different concentrations (2, 5, 10 or 20 µg/ml). The data are mean of five consecutive parallel readings presented in Table 2.

IC_{50} values for 50% LPI activity was 15.7 µg extract ± 2.17 (*A. awasthii*), 12.68 µg extract ± 1.32 (*H. podocarpa*), and 11.47 µg extract ± 0.68 (*P. tinctorum*). IC_{50} of these three culture extracts for EDA were found to be 13.23 µg extract ± 1.14 (*A. awasthii*), 18.37 ± 2.08 (*H. podocarpa*) and 13.64 ± 1.7 (*P. tinctorum*) respectively. IC_{50} for TI activity 8.71 µg extract ± 0.83 (*A. awasthii*), 14.55 µg extract ± 0.33 (*H. podocarpa*), and 12.44 µg extract ± 0.17 (*P. tinctorum*) in the assay system was found less than standard Trolox or kojic acid.

DISCUSSION

In this study we could clearly prove lipid peroxidation inhibition effects, electron donating ability and tyrosinase inhibitory potential in the methanolic extracts of cultured symbionts from the lichen species *A. awasthii*, *H. podocarpa,* and *P. tinctorum*. Lichens produce a number of secondary metabolites polysaccharides or phenolic com -pounds that are known to exhibit such properties (Liu et al., 1997, Hidalgo et al., 1994, Sanchez-Moreno et al., 1999). Our results are in agreement with those that reported the ability of phenolic and polysaccharides scavenge free radicals and active oxygen species (Germano et al., 2002; Duh et al., 1999; Okamoto et al.,1992) and for tyrosinase inhibitory activity (Suzuki et al., 1992).

The function of secondary metabolic compounds in cultures depends on various internal and external factors these includes nutritional requirements, ecological factors and stress situations. A complex interrelationship of these factors was found to influence the induction of secondary pathways and the production of specific lichen substances in culture (vide Stocker-Wörgötter and Elixir, 2002). Furthermore the major metabolites are frequently accompanied by so-called satellite compounds which are biochemically related intermediate- and side products (Elix, 1996).We have analyzed the extracts to know the group of phenolic compounds present in the extract. The extract of cultured symbionts of lichen species *A.*

awasthii, *H. podocarpa* and *P. tinctorum* showed the presence of ihydroxy and monohydroxy phenolic groups. Levels of phytochemicals are influenced by genetics and growth conditions (Marin et al., 2004). At present we can say only that the biological activities reported here are species specific.

The present results indicated that the methanol extract of cultured symbionts of lichen species *A. awasthii*, *H. podocarpa* and *P. tinctorum* can be used as alternative to synthetic tyrosinase inhibitor. However, further study on the toxicity of the bioactive component present in the extract is highly required before pharmaceutical use. If lichen cultures are to have industrial use then we must improve their growth rate (Brunauer and Stocker-Worgotter, 2005).

ACKNOWLEDGEMENTS

We are very grateful to the Department of Biotechnology, Government of India, New Delhi, for the financial support. We also gratefully acknowledge financial support in the form of a Senior Research Fellowship to Neeraj Verma from Council of Scientific Industrial Research, New Delhi, India. We are also thankful to Dr. D. G. Naik, Chemistry Group, Agharkar Research Institute, for providing the HPLC facility.

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