Growth promoting effects of some lichen metabolites on probiotic bacteria

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Abstract In the present study, the extract of four natural lichen species *Canoparmelia eruptens*, *Everniastrum cirrhatum*, *Parmotrema austrosinense* and *Rimelia cetrata* were studied for the source of natural antioxidant and their purified secondary metabolites were evaluated for growth promoting effects on probiotic bacteria *Lactobacillus casei*. The methanolic fraction of lichen species showed moderate to high antioxidant activity in the order *P. austrosinense* > *E. cirrhatum* > *C. eruptens* > *R. cetrata*. The lichen metabolites showed antioxidant activity with an IC_{50} values ($\mu\text{g/ml}$); lecanoric acid 79–95, salazinic 88–108, atranorin 100–116 and consalazinic acid 119–125. As far as the growth promoting effects of lichen metabolites on *L. casei* is concerned, lecanoric acid at 100 $\mu\text{g/ml}$ conc. showed high growth stimulating activity in terms of increased dry matter of biomass (56.08 mg) of *L. casei*. Other lichen metabolites; salazinic acid, atranorin and consalazinic acid produced relatively less dry biomass 43.98 mg, 41.1 mg, 40.68 mg, respectively. However, standard antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and Trolox after 36 h produced 39.04–47.81 mg dry biomass. At lower pH the growth promoting activity of lichen metabolites was found stable.

Keywords Lichen metabolites · Antioxidant activity · Probiotic potential

Introduction

In the human intestinal tract a great number of bacteria exists, which are beneficial and known as probiotic bacteria. These bacteria are useful in nutrient synthesis and improve the digestibility of dietary nutrients like proteins and fat. A number of studies suggested that consumption of probiotic bacteria; *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Lactococcus* and *Saccharomyces* as a food supplement improves the immune system and reduce the risk of many diseases like colon cancer, diarrhea and allergies (Grajek et al. 2005). In order to reduce such diseases a high number of potential probiotic bacteria are needed to be supplemented with the food products.

Nowadays major concern in food industries are oxidation of lipids in food products. This causes rancid odors and flavors of the products by the formation of potentially toxic compounds in consequence, decrease in nutritional quality and safety. The problem of ensuring a high quality of lipids in lipid-containing products and prolonging their storage time is directly associated with their optimum stabilization by addition of suitable antioxidants (Yanishlieva et al. 2006). Antioxidants are also very important to human health protection as they scavenge harmful free radicals or hinder the oxidative processes of biomolecules (lipid, protein, DNA, carbohydrates etc.) and thereby prevent various types of degenerative diseases including cancer (Arnao et al. 2001).

To ensure health benefits, the use of probiotic bacteria and antioxidants are now became an important part of our functional food (Grajek et al. 2005). In a recent report, Duda-Chodak et al. (2008) suggested that antioxidants play a growth promoting role in probiotic bacteria cultured in vitro. In food industries synthetic antioxidants; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are most extensively

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used (Sherwin 1990). However, due to the suspected toxicity of synthetic antioxidants used in food products has raised the concern and thereby many researchers have focused on the discovery of new natural antioxidants for promoting of probiotic potential of bacteria especially from plants (Hettiarachchy et al. 1996; Bouaziz and Sayadi 2005; Aligiannis et al. 2003; Es-Safi et al. 2007).

Lichens are unusual organisms composed of a fungus (mycobiont) with at least one symbiotic photosynthetic partner (photobiont). They are proven as the earliest colonizers of terrestrial habitats on earth with a worldwide distribution from arctic to tropical regions and from the plains to the highest mountains (Taylor et al. 1995; Mitrović et al. 2011). Due to living in extreme environmental conditions lichen developed various compounds for their survival, which are not known to occur in any other organism in nature (Huneck and Yoshimura 1996). These lichen secondary metabolites are mainly phenolic compounds; depsides, depsidones, dibenzofuranes and other accessory pigments (Nash 1996). Throughout the ages, lichens and their secondary compounds are used for different purposes for humans and animals depending on their nutritive, medicinal, decorative, brewing, distilling, dying, cosmetic and perfumery properties (Richardson 1988; Upreti and Chatterjee 2007; Karagoz et al. 2009). Till date 1050 lichen compounds are reported out of which some of them have shown various biological actions; antibiotic, antimycobacterial, antiviral, antitumour, analgesic, antipyretic and enzyme inhibitory (Matsubara et al. 1997; Huneck 1999; Müller 2001; Behera et al. 2006; Oksanen 2006; Lopes et al. 2008; Molnár and Farkas 2010).

In our literature survey we could not find any report that explains lichen extract or their metabolites have antioxidative along with probiotic potential. Therefore, in the study we have investigated the antioxidant activity and probiotic potential of purified lichen secondary metabolites.

Materials and methods

Chemicals

Linoleic acid, 1,1-diphenyl-2-picryl hydrazil (DPPH), potassium hexacyanoferrate (III), folin-ciocalteu reagent, peroxidase and polysaccharide soln. were procured from Hi-Media Chemicals, India. Other chemicals like 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Chemical, USA. All other routine chemicals/culture media used were of analytical grade.

Lichen material

Fresh thallus of natural lichen species *Canoparmelia eruptens* (Kurok.) Elix & Hale, *Everniastrum cirrhatum* (Fr.) Hale ex Sipman, *Parmotrema austrosinense* (Zahlbr.) Hale and *Rimelia cetrata* (Ach.) Hale & Fletcher have been collected from Nilgiri hills, Tamilnadu State, India. Lichen species were identified on the basis of their morphology and anatomy. Sections of lichen thalli and ascomata were mounted in water, 10 % KOH (K), Lugol's solution (I) and lactophenol cotton-blue (LPCB). Then all the anatomical measurements were made on material mounted using a compound microscope (Nikon Eclipse 80I). Lichen substances were identified by standardized method of TLC (Culberson and Kristinsson 1970) and HPLC (Feige et al. 1993). In HPLC lichen substances were identified by their peak symmetry and their retention time (r_t), by comparison with authentic substances made to standard concentration and compared with the literature for the ultraviolet spectrum of the lichen metabolites (Huneck and Yoshimura 1996; Yoshimura et al. 1994). The retention times (rt) recorded for atranorin (2.21 min), lecanoric acid (7.49 min), salazinic acid (5.07 min) and consalazinic acid (2.13 min). A part of fresh thallus of natural lichens *C. eruptens* producing atranorin and lecanoric acid (voucher no. AMH 99.370); *E. cirrhatum* producing salazinic acid and atranorin (voucher no. AMH 99.371); *P. austrosinense* producing atranorin and lecanoric acid (voucher no. AMH 99.372) and *R. cetrata* producing salazinic acid, consalazinic acid and atranorin (voucher no. AMH 99.369) have been preserved as specimen in the Ajrekar Mycological Herbarium (AMH) at Agharkar Research Institute, Pune, India.

Lichen species extraction

The natural thallus of lichens were separated from bark of the tree and washed by keeping over night under flow of tap water and then by distilled water. Cleaned thallus was cut into small pieces and extracted successively in Soxhlet apparatus with different organic solvents; *n*-hexane, chloroform, ethyl acetate, acetone, methanol and dimethyl sulphoxide (DMSO) by soaking. The resulted fractions were filtered by Whatman filter paper no.1, dried *in vacuo* and stored at 4 °C until use. The quantities of dry fractions obtained from different solvents are given in Table 1.

Phytochemical content estimation of lichen species

Lichens produce many primary and secondary compounds for their different metabolic activities. In order to know the quantity and the compound responsible for the observed antioxidant activity, total phenolics,

Table 1 Dry extract and phytochemical contents obtained from fractionation extraction of natural lichen thallus in various solvents

Lichen Species	Dry extract (gm)	Total polyphenol (mg/gm dry wt.)	Protein (mg/gm dry wt.)	Polysaccharide (mg/gm dry wt.)
<i>C. eruptens</i>				
n-Hexane	0.034	0.38±0.05	0.15±0.04	1.26±0.12
Chlf	0.034	0.45±0.01	0.66±0.07	1.38±0.25
EtoAc	0.119	1.08±0.02*	0.82±0.02	1.98±0.68
Acet	0.092	0.81±0.08	0.74±0.03	1.66±0.62
MeOH	0.172*	1.10±0.06**	1.37±0.24*	2.09±0.93*
DMSO	0.549**	0.12±0.03	1.40±0.33**	5.09±1.02**
<i>E. cirrhatum</i>				
n-Hexane	0.014	0.13±0.04	0.34±0.02	1.46±0.23
Chlf	0.021	0.16±0.03	0.35±0.01	1.81±0.16
EtoAc	0.042	0.24±0.01*	1.08±0.30	2.59±0.32
Acet	0.028	0.22±0.05	0.60±0.02	2.34±0.80
MeOH	0.069*	0.33±0.01**	1.17±0.13*	3.13±0.76*
DMSO	0.640**	0.10±0.03	1.71±0.21**	4.24±0.98**
<i>P. austrosinense</i>				
n-Hexane	0.019	0.45±0.03	0.35±0.02	1.85±0.19
Chlf	0.022	0.57±0.04	0.38±0.04	2.37±0.42
EtoAc	0.044	1.10±0.06*	0.67±0.05	2.40±0.63
Acet	0.044	0.87±0.02	0.55±0.03	2.40±0.75
MeOH	0.095*	1.34±0.04**	0.77±0.04*	4.57±0.31*
DMSO	0.563**	0.43±0.07	1.30±0.10**	5.20±1.10**
<i>R. cetrata</i>				
n-Hexane	0.019	0.25±0.03	0.23±0.02	1.26±0.10
Chlf	0.036	0.25±0.04	0.24±0.02	2.42±0.21
EtoAc	0.114	0.60±0.02*	0.61±0.03	2.98±0.53
Acet	0.071	0.37±0.05	0.56±0.07	2.45±0.76
MeOH	0.126*	0.63±0.01**	1.52±0.08*	3.70±0.12*
DMSO	0.313**	0.14±0.03	1.54±0.21**	4.13±0.95**

Chlf Chloroform; EtoAc Ethyl acetate; Acet Acetone; MeOH Methanol; DMSO Dimethyl sulphoxide. Phytochemical content results presented are the mean (±SD) of the three parallel measurements ($n=3$). Value of * $p<0.05$ were regarded as significant and ** $p<0.01$ as very significant

polysaccharide and protein content present in various extract fractions of lichen species were estimated. Total polysaccharide content was determined, using the phenol-sulfuric acid method of Dubois et al. (1956). Protein content was determined by the method of Lowry et al. (1951). The protein content was estimated by a standard curve using known amount of bovine serum albumin (BSA). Total soluble phenolic content was determined with Folin-Ciocalteu reagent according to Slinkard and Singleton (1977), using pyrocatechol as a standard. Details of all the assays are reported earlier by us (Behera et al. 2005; Verma et al. 2008).

Phenolic antioxidant group detection by TLC

In order to know the nature of compounds present in the lichen extract responsible for the antioxidant activity, TLC bioautography assay was performed according to the procedure previously described (Behera et al. 2009).

Antioxidative potential of lichen species

DPPH radical-scavenging assay

The DPPH radical-scavenging (DRS) activity of lichen fractions was measured by 1,1-diphenyl-2-picryl hydrazil according to Blois (1958) with minor modifications (Verma et al. 2008). The standard antioxidants (50 µg/ml) of each; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and Trolox, a water-soluble vitamin E analogue were used as positive control. A decrease in absorbance of the reaction mixture indicated a higher free radical scavenging activity. DPPH radical scavenging was expressed as the inhibition percentage and calculated using the following formula of Yen and Duh (1994):

$$\% \text{ Inhibition} = [(A_{\text{DPPH}} - A_{\text{Extr}})/A_{\text{DPPH}}] \times 100$$

Where A_{DPPH} is the absorbance value of the DPPH blank sample, and A_{Extr} is the absorbance value of the test solution.

Anti-linoleic acid peroxidation assay

The anti-linoleic acid peroxidation (ALP) activity of lichen fractions was determined according to Liegeois et al. (2000). Conjugated diene hydroperoxides formation with or without lichen extract was monitored by increase in absorbance at 234 nm. Standard antioxidants (BHA, BHT, Trolox) were used as positive control. The percentage inhibition of lipid peroxidation was calculated by the following equation:

$$\% \text{ Inhibition} = ([A_0 - A_1]/A_0) \times 100$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the extract.

Trolox-equivalent antioxidant capacity assay

Trolox-equivalent antioxidant capacity (TEAC) of lichen fractions was determined according to Miller et al. (1995) with minor modifications (Verma et al. 2008). The TEAC value is based on the ability of the antioxidant to scavenge the blue-green 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) ($\text{ABTS}^{•+}$) radical cation relative to the $\text{ABTS}^{•+}$

scavenging ability of the water soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The decrease in absorbance at 734 nm after the addition of reactants was used to calculate the TEAC value. The TEAC value was expressed as mM of Trolox solution having the antioxidant equivalent to a 0.1 % (w/v) extract solution.

Isolation and purification of lichen metabolites

The methanol fraction of lichen species were dried *in vacuo* and then used for isolation of lichen metabolites by preparative TLC. Silica coated TLC plates spotted with extracts were eluted several times by TDA (toluene: 1-4-dioxane: acetic acid; 180 ml: 45 ml: 5 ml) to obtain pure atranorin, lecanoric acid, salazinic acid and consalazinic acid. The lichen metabolites were further centrifuged at 10,000 rpm for 10 min and the supernatant was subjected to HPLC analysis.

Antioxidant activity of isolated metabolites

The antioxidant activity of purified lichen metabolites at three different concentrations; 50, 100 and 200 µg/ml were determined using the above mentioned *in vitro* antioxidant assays. The half inhibitory concentration (IC₅₀) values were calculated by extrapolation from concentration/effect regression lines obtained from the above mentioned concentrations.

Growth promoting effects of lichen metabolites on probiotic bacteria

Microorganism

Bacterial strain *Lactobacillus casei* (NCIM 2737) was procured from National Collection of Industrial Microorganisms at National Chemical Laboratory, Pune, India and maintained on recommended MRS medium. To determine the suitable concentration of *L. casei* for the study of growth promoting effects of lichen metabolites, bacterial culture was serially diluted in the concentration of 10⁻², 10⁻⁴, 10⁻⁶ and 10⁻⁸ in MRS broth medium and kept at 37 °C for 24 h. The resulting 10⁻⁸ concentration having OD₅₅₀=0.2 was selected for the experiments.

Effects of lichen metabolites on the growth of L. casei

The growth promoting effects of lichen metabolites on *L. casei* was evaluated on the basis of dry matter of biomass obtained from cultures at different time intervals. Lichen metabolites atranorin, lecanoric acid, salazinic acid and consalazinic acid of 50, 100 and 200 µg/ml conc. prepared in methanol were individually added to the test tubes

containing 10 ml sterile MRS broth media. The resulted solutions were vortexed and then inoculated with 100 µl of bacterial suspension (10⁻⁸). MRS broth media having bacterial suspension and methanol was used as negative control. Standard antioxidant BHA, BHT and Trolox were used as positive control. The experiments were performed in triplicate and the mean values were used for calculations. As Duda-Chodak et al. (2008) suggested the number of bacteria is directly proportional to the dry bacterial biomass. Therefore, the increase in bacteria number causes an adequate rise of the dry biomass. After 24, 36 and 48 h incubation period, the produced biomass of *L. casei* was harvested by centrifugation at 10,000 rpm and pellet of bacteria was dried at 100 °C in a water bath and then weighed.

Effects of pH and metabolites on growth of L. casei culture

At various stages of gastrointestinal system, the pH varied greatly (1.0–6.5). However, the majority of nutrients, vitamins and drugs are absorbed in duodenum where the pH is 6 to 6.5. Recently Duda-Chodak et al. (2008) reported that *Lactobacillus* cultures decreases the pH and changes can cause transformation of antioxidants present in the raw materials into the forms of lower or higher antioxidant potential. Therefore, experiments were conducted to check the stability of antioxidant activity in terms of growth enhancement potential of lichen metabolites for *L. casei* culture at different pH ranges. Lichen metabolites atranorin, lecanoric acid, salazinic acid, consalazinic acid at 100 µg/ml concentrations were prepared in 10 mM phosphate buffer of pH 5, 6.5 and 8, and then incubated at 37 °C for 1 h. The growth promoting activity of lichen metabolites were measured after 36 h by estimating the dry bacterial biomass produced.

Statistical analysis All the results expressed are mean (±SD) of the three parallel measurements (*n*=3). Statistical significance was evaluated by Sigma Stat Version 9.0. Value of *p*<0.05 were regarded as significant and *p*<0.01 as very significant. The correlation between phytochemical contents and antioxidant activities were determined by plotting linear regression graph using Sigma Plot Version 11 and the *r*² value was calculated.

Results and discussion

Antioxidative potential of lichen species *C. eruptens*, *E. cirrhatum*, *P. austrosinense* and *R. cetrata* fractionated in various organic solvents were determined in terms of DRS, ALP and TEAC activity. The methanol, ethyl acetate and acetone solvent fractions showed DRS activity as *C.*

eruptens was 55.38 to 63.64 %, *E. cirrhatum* 67.95 to 74.10 %, *P. austrosinense* 57.50 to 82.27 % and in *R. cetrata* 40.24 to 44.85 %. These activities were found less or equivalent to that of standard antioxidants BHA, BHT (76.2 and 82.1 %) (Fig. 1). Methanol, ethyl acetate and acetone fractions of lichen species showed ALP activity. Methanol fraction of *P. austrosinense* (56.43 %) showed higher activity than

E. cirrhatum (50.88 %), *C. eruptens* (42.19 %), *R. cetrata* (30.41 %) and were found less or equivalent to the activity of standard BHA, BHT and Trolox (37.1 to 55.4 %). As far as the TEAC of lichen species is concerned, methanol, ethyl acetate and acetone fractions of *P. austrosinense* gave 5.67, 4.50, 4.42 mM; *E. cirrhatum* 4.24, 3.40, 1.84 mM; *C. eruptens* 3.72, 1.85, 1.13 mM and *R. cetrata* 3.35, 2.39,

Fig. 1 DPPH radical scavenging activity, Anti-linoleic acid peroxidation activity and Trolox equivalent antioxidant capacity of various solvent fractions of lichen species and standard antioxidants at 50 µg/ml conc. Results presented are the mean (±SD) of the three parallel measurements ($n=3$). Value of $*p<0.05$ were regarded as significant and $**p<0.01$ as very significant. BHA: Butylated hydroxyanisol, BHT: Butylated hydroxytoluene. Chlf: Chloroform; EtoAc: Ethyl acetate; Acet: Acetone; MeOH: Methanol; DMSO: Dimethyl sulphoxide

2.09 mM, respectively. However, these TEAC values were found more or equivalent to the standard Trolox (3.6 mM) (Fig. 1). In general the order of antioxidant activities in lichen species was observed as *P. austrosinense* > *E. cirrhatum* > *C. eruptens* > *R. cetrata*. These results proved the studied lichen species can be a good source of natural antioxidants.

The phytochemical contents; protein, polysaccharide and polyphenol present in various solvent fractions of lichen species are given in Table 1. High polyphenolic content was found in methanol fraction of lichen species *P. austrosinense* (1.34 mg), *C. eruptens* (1.10 mg), *E. cirrhatum* (0.33 mg) and *R. cetrata* (0.63 mg). Whereas, low polyphenol content was obtained in DMSO fraction. As far as polysaccharide and protein content are concerned, DMSO fraction of lichen species had a maximum amount of polysaccharide (4.13–5.20 mg/gm) and protein content (1.30–1.71 mg/gm) followed by methanol, ethyl acetate, acetone, chloroform and *n*-hexane fractions.

Antioxidant activity of lichen species in relation to phytochemical content showed high significant correlation with the polyphenol content; DRS' $r^2=0.8221$, ALP' $r^2=0.8664$ and with TEAC' $r^2=0.7332$. While a non significant correlation ($r^2<0.33$) was found with polysaccharide and protein content. These results were supported by the TLC bioautography analysis, where plate a) sprayed with DPPH soln. gave yellow spot, indicating the presence of antioxidant compounds; plate b) sprayed with Barton's reagent gave blue colour spots, indicating the presence of phenolic compounds and plate c) sprayed with ferric chloride gave blue spots, confirms the presence of phenolic compound of trihydroxy group along with reddish brown colour spots for other phenolics in *C. eruptens*, *R. cetrata*, *E. cirrhatum* and *P. austrosinense*. Our results are in agreement with those reported that scavenging ability of phenols is due to their hydroxyl group (Hatano et al. 1989; Duh et al. 1999).

The purified lichen metabolites showed antioxidant activities are presented in Fig. 2. Lecanoric acid at 100 µg/ml concentration showed 53 to 64 % activity, salazinic acid 46.4 to 57.2 %, atranorin 43.3 to 50.3 % and consalazinic acid 40 to 42.3 % activity. While at 50 and 200 µg/ml concentration, the antioxidant activity was observed ≤40 %. As far as TEAC of lichen metabolites are concerned, the TEAC value of lecanoric acid at 100 µg/ml was found 3.1 mM, which was higher than salazinic acid 2.5 mM, atranorin 2.1 mM and consalazinic acid 1.6 mM. The IC₅₀ value of lichen metabolites for the antioxidant activity showed that lecanoric acid was most active at 79 to 95 µg/ml, followed by salazinic 88 to 108 µg/ml, atranorin 100 to 116 µg/ml and consalazinic acid 119 to 125 µg/ml. These IC₅₀ concentrations of lichen metabolites were found more or less equal to the standard antioxidants BHA, BHT and Trolox (61–135 µg/ml) (Table 2).

Fig. 2 DPPH radical scavenging activity (DRS), Anti-linoleic acid peroxidation activity (ALP) and Trolox equivalent antioxidant capacity (TEAC) of lichen metabolites at 100 µg/ml conc. Results presented are the mean (±SD) of the three parallel measurements ($n=3$). Value of * $p<0.05$ were regarded as significant and ** $p<0.01$ as very significant

The growth promoting effects of lichen metabolites on *L. casei* culture are presented in Fig. 3. Lecanoric acid at 100 µg/ml conc. showed high growth stimulating activity in terms of increased dry matter of biomass (56.08 mg) of *L. casei* in comparison to the negative control (31.79 mg) and positive control with standard antioxidants BHA, BHT and Trolox (39.04–47.81 mg) after 36 h. At the same concentration of salazinic acid, atranorin and consalazinic acid produced relatively less dry matter of biomass 43.98 mg, 41.1 mg, 40.68 mg, respectively. This biomass content was found lower than the standard antioxidants. In our study, no growth of *L. casei* was observed after 24 h in BHA and BHT supplemented cultures. The effects of lower/higher concentrations (50, 200 µg/ml) of lichen metabolites on the growth of *L. casei* at 24 h or 48 h were varied. As far as the effects of pH on growth promoting activity of lichen metabolites is

Table 2 Half-inhibiting concentration (IC₅₀) of lichen metabolites for the antioxidant activity

Lichen metabolites	Half-inhibiting concentration (IC ₅₀ , µg/ml)	
	DRS	ALP
Atranorin	100	116
Consalazinic acid	119	125
Lecanoric acid	79	95
Salazinic acid	88	108
Standard antioxidants		
BHA	66	135
BHT	61	98
Trolox	–	91

DRS DPPH radical scavenging activity; ALP Anti-linoleic acid peroxidation activity; BHA Butylated hydroxyanisole, BHT Butylated hydroxytoluene. Data presented are the mean of three consecutive readings ($n=3$) of the sample in assay performed

Dry biomass of *L. casei* (mg)

Fig. 3 Effects of (a) standard antioxidants (100 µg/ml) and (b) lichen metabolites (100 µg/ml) on the growth of *L. casei* at various time intervals. BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene. Results presented are the mean (±SD) of the three parallel measurements ($n=3$)

concerned, lecanoric acid and salazinic acid were found most stable at pH range 5 to 8 with the production of dry biomass of *L. casei* 52.7 to 54.3 mg and 37.6 to 38.9 mg, respectively (Fig. 4). While atranorin and consalazinic acid showed 36.4 to 29.4 mg and 30 to 22.6 mg dry biomass content at same range of pH. These results suggested that at lower pH 5.0, lichen metabolites can retain their probiotic potential in terms of promoting the growth of *L. casei*.

Many of the lichen extracts and their metabolites are reported to have variety of biological actions including antioxidative potential (Müller 2001; Kosanić et al. 2011; Kosanić and Ranković 2011). Very recently Ahire et al. (2011) reported that antioxidant activity of intracellular

Fig. 4 Effects of different pH on growth enhancement activity of lichen metabolites for *L. casei* culture after 36 h. Results presented are the mean (±SD) of the three parallel measurements ($n=3$). Value of $*p<0.05$ were regarded as significant and $**p<0.01$ as very significant

cell-free extract of a *Lactobacillus* species. In our study supplementation of lichen metabolites significantly enhanced the growth of *L. casei*. Therefore, it can be hypothesized that the lichen metabolites are involved in some kind of synergistic action with the intracellular component of *L. casei*. However this synergistic effect is yet to be demonstrated. As there is a great demand of natural antioxidants and probiotics in food and pharmaceutical industries to prolong the stability and storage life of food products (Martínez-Tomé et al. 2001; Das et al. 2012). These lichen metabolites can be of help as supplement for promoting the nutritional value of the food products.

Conclusion

In the present study, the lichen secondary metabolites lecanoric acid, salazinic acid, atranorin and consalazinic acid showed antioxidant activity along with probiotic potential in terms of stimulating the growth of *L. casei*. These results inferred that lichen metabolites may be used as nutraceutical supplements in the food products to reduce the oxidative stress related diseases. However, there is a requirement to explore them as nutraceutical products by executing pre clinical trials. This investigation provide as additional information on probiotic properties of the lichen metabolites which are not reported earlier.

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