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BIOLOGICAL EVALUATION AND PHYTOCHEMICAL PROFILING OF SOME LICHEN SPECIES

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Lichens are a symbiotic relationship between a fungus and a photosynthetic partner. Chemical characterization and bioactive potentials (antiproliferative, antioxidant, and antibacterial) of five lichen species (*Evernia prunastri*, *Platismatia glauca*, *Pseudevernia furfuracea*, *Ramalina fastigiata*, and *Ramalina farinacea*) were assessed. Five lichen metabolites (usnic acid, atranorin, stictic acid, evernic acid, and fumarprotocetraric acid) were analyzed by HPLC-DAD. *E. prunastri* was noteworthy evernic acid source. Antiproliferative activity was evaluated using human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines. The strongest activity was observed for *P. glauca* against HepG2/C3A, while the only lichen species that induced cytotoxicity against MCF-7 cell line was *P. furfuracea*. The highest antioxidant activity was also obtained with *P. furfuracea*. *E. prunastri* and *R. farinaceae* had the highest phenolic and flavonoid contents, respectively. Antibacterial activities of the extracts were determined against ten pathogenic bacteria. The most effective antibacterial agent was methanol extract of *R. fastigiata*. Our findings have revealed the pharmaceutical potentials of tested lichen species.

Keywords: antibacterial, antioxidant, antiproliferative, chemical profile, HPLC, lichen

It is estimated that about 18 000–25 000 lichen species are colonized in the world (Sipman & Aptroot, 2001; Chapman, 2009). They have been used traditionally as medicine by Chinese people and Native Americans for hundreds of years. They are also used as food source, natural dyes, as well as in the alcohol and perfume industries (Kosanic et al., 2013).

More than a thousand different secondary metabolites have been reported for lichen species and their mycobionts (Molnár & Farkas, 2010). Previous studies indicate that lichen secondary metabolites exert a broad range of biological activities that comprise antibiotic, antiviral, antimycobacterial, antiinflammatory, analgesic, enzyme inhibitory, antipyretic, and cytotoxic effects (Kosanic et al., 2013; Korkmaz et al., 2018). While the potential of the numerous lichen compounds was elucidated, many promising candidates still need to be investigated.

The present study aims to assess the antioxidant, antibacterial, and antiproliferative potentials of five lichen species and to elucidate their chemical contents by HPLC-DAD as possible therapeutic agents.

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1. Materials and methods

1.1. Lichen material and extraction

Five different lichen species [*Evernia prunastri* (L.) Ach., *Platismatia glauca* (L.) W.L.Culb. & C.F.Culb., and *Pseudevernia furfuracea* (L.) Zopf. from Parmeliaceae; *Ramalina fastigiata* (Pers.) Ach. and *Ramalina farinacea* (L.) Ach. from Ramalinaceae families] were collected from several provinces (Lake Abant province, Aladağlar province, and Lake Seben province) of Bolu, Turkey between 2014–2015. Extraction procedures were performed according to the method described by Tas and co-workers (2017).

1.2. High-performance liquid chromatography (HPLC-DAD) analysis

HPLC-DAD analysis was conducted according to the method described by Kosanic and co-workers (2014).

1.3. Antiproliferative activity

Antiproliferative activity of acetone extracts of tested lichens against human breast adenocarcinoma (MCF-7) and human hepatocellular carcinoma (HepG2/C3A) cell lines were determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) colorimetric assay (Mosmann, 1983) according to the procedure described by Tas and co-workers (2017).

1.4. Free radical scavenging activity

Free radical scavenging activity of methanol extracts of lichen species was evaluated by using 2,2-diphenyl-1-picrylhydrazil (DPPH•, Sigma®) radical photometric assay according to Blois (1958)'s method modified by Tas and co-workers (2017).

1.5. Determination of total phenolic and flavonoid contents

Phenolic and flavonoid contents in methanol and acetone extracts of lichens were determined by using the Folin–Ciocalteu (Slinkard & Singleton, 1977) and aluminium chloride ($AlCl_3$) colorimetric assays (Zhishen et al., 1999), respectively, with some modifications described by Tas and co-workers (2017).

1.6. Antibacterial bioassay

Antibacterial effect of lichen extracts was investigated against ten pathogenic bacteria by using disc diffusion method (Andrews, 2009) with some modifications described by Tas and co-workers (2017).

1.7. Statistical analysis

Data analysis was conducted by analysis of variance (ANOVA) and Duncan's multiple range tests using SPSS vers. 15 (SPSS Inc, Chicago, IL, USA).

2. Results and discussion

Chemical constituents of tested lichen species and extraction yields are shown in Table 1. HPLC-DAD analysis showed that acetone was more efficient solvent for obtaining lichen metabolites (Table 1). The chromatogram of the standards and each analyzed lichen species are demonstrated in Figures 1 and 2, respectively.

Table 1. Tested lichen species and quantitative analysis of extracts by HPLC-DAD

Lichen species	Extract	Yield (%)	Standard compounds (mg g^{-1} dry extract)				
			Atranorin	Evernic acid	Usnic acid	Stictic acid	Fumarprotocetraric acid
E. prunastri	AE	7	102.27±0.17	258.81±0.43	22.20±1.10	–	–
	ME	8.6	10.09±0.07	111.99±0.19	22.82±0.82	–	–
P. glauca	AE	2.2	59.88±0.02	–	–	–	–
	ME	3.3	11.31±0.38	–	–	–	–
P. furfuracea	AE	3.7	115.98±0.29	–	–	–	–
	ME	8.2	3.03±0.02	–	–	–	–
R. fastigiata	AE	1.4	–	112.9±0.92	65.24±0.02	–	–
	ME	4	–	41.5±0.00	57.76±1.00	–	–
R. farinacea	AE	3	–	–	57.61±0.14	–	–
	ME	4.8	–	–	38.02±0.07	–	–

Data are presented as mean lichen acid amount±standard error (SE). AE: acetone; ME: methanol

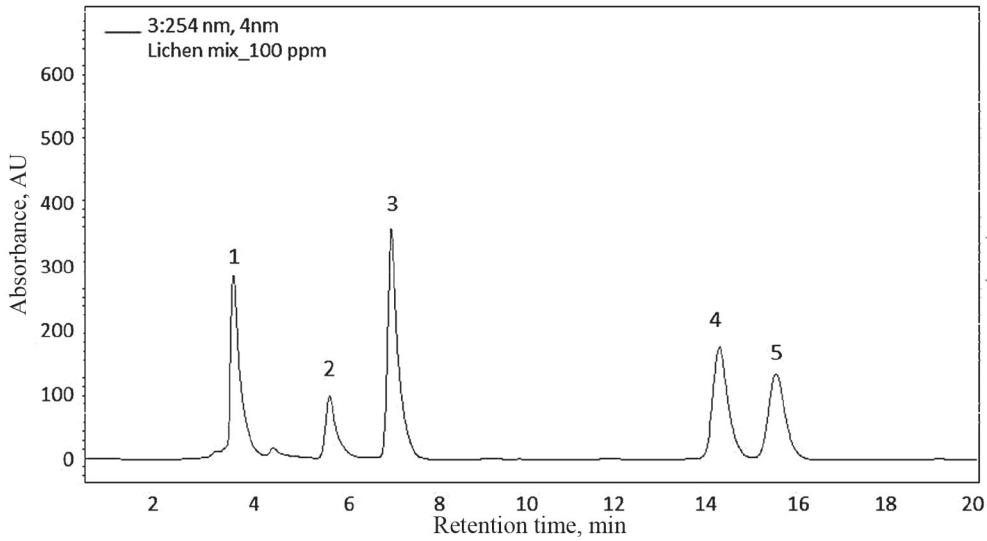

Fig. 1. Chromatogram of standards used for identification. Retention times: 1. stictic acid-3.47 min; 2. fumarprotocetraric acid-5.42 min; 3. evernic acid-6.77 min; 4. usnic acid-13.70 min; and 5. atranorin-14.87 min

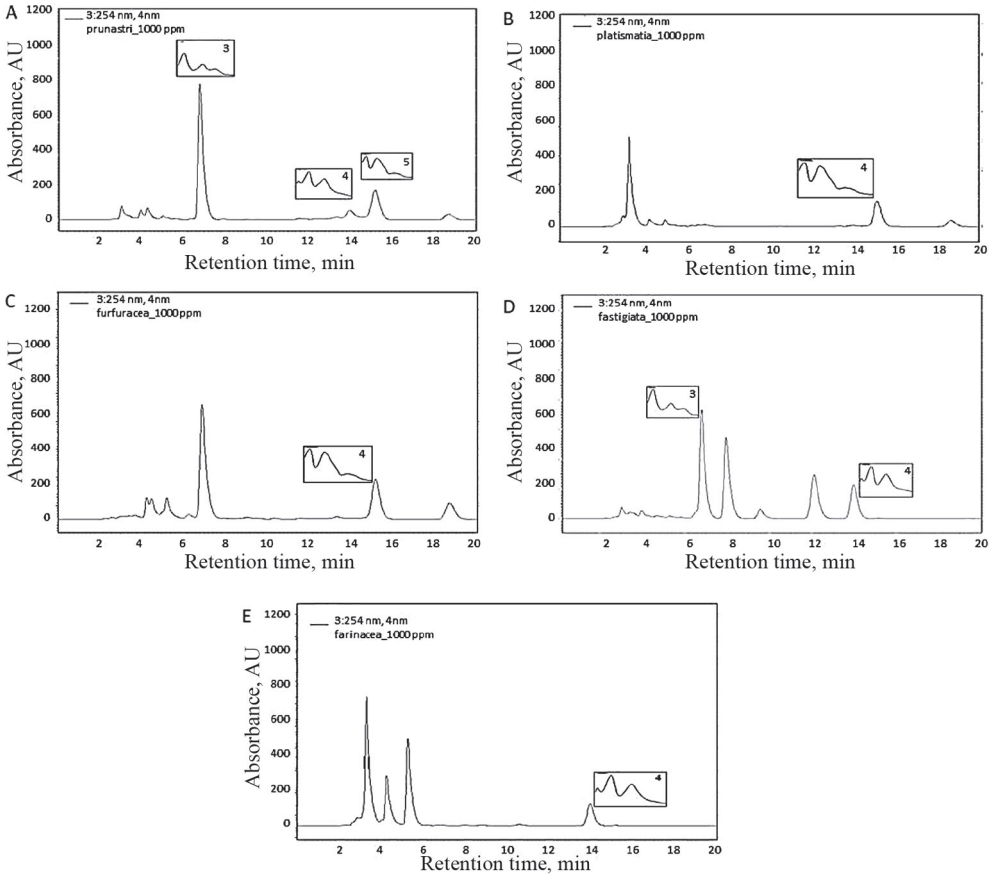

Fig. 2. HPLC chromatograms acquired at 254 nm of the acetone extract of tested lichen species. (A) *E. prunastri*, (B) *P. glauca*, (C) *P. furfuracea*, (D) *R. fastigiata*, and (E) *R. farinacea*

In consistence with the previous study (Kosanic et al., 2013), evernic acid was the main compound in acetone extract of *E. prunastri*. Only atranorin was detected in the acetone extract of *P. glauca* in our study. *P. glauca* showed higher atranorin concentration compared to 2.62 mg g^{-1} of atranorin in the study of Nybakken and co-workers (2010). Moreover, acetone extract of *P. furfuracea* had the highest atranorin amount (Table 1). Similar to the result of Kosanic and co-workers (2013), the main compound of acetone extract of *P. furfuracea* was atranorin. Evernic acid and usnic acid were found in the acetone extract of *R. fastigiata* (Table 1). It was the second best evernic acid source after *E. prunastri.* Consistently with the report of Ristic and co-workers (2016), *R. fastigiata* contained the most considerable usnic acid amount. We similarly reported that *R. farinacea* was a source of usnic acid (Tay et al., 2004). Previous studies showed that evernic acid, atranorin, and usnic acid had antimicrobial, antioxidant, anti-inflammatory, and anticancer activities (Odabasoglu et al., 2006; Turk et al., 2006; Molnár & Farkas, 2010; Kosanic et al., 2014; Zhou et al., 2017).

Antiproliferative effects of acetone extracts were evaluated by MTT assay against MCF-7 (human breast cancer) and HepG2/C3A (human hepatocellular carcinoma) cell lines. HepG2/

C3A cell line was more vulnerable to lichen extracts. The highest effect was observed for *P. glauca*, followed by *R. farinacea* against HepG2/C3A cell line. The only lichen species that was active against both cell lines was *P. furfuracea* (Table 2).

Until now, few researches have evaluated the cytotoxic properties of selected lichen species. Secondary metabolites of *P. furfuracea*, olivetoric acid and pysodic acid, showed cytotoxicity against glioblastoma cell line U87MG (Emsen et al., 2016). Anticancer activity of *E. prunastri* and *P. furfuracea* was determined against human melanoma (FemX) and colon carcinoma cell (LS174) (Kosanic et al., 2013). Acetone extract from *R. fastigiata* exhibited potent anticancer activity against Hela, A549 and LS174 cell lines (Ristic et al., 2016). In one study, *n*-hexane, diethylether, and methanol extracts of *E. prunastri* and *P. glauca* demonstrated antiproliferative activity against MCF-7 cell line (Bézivin et al., 2003). However, to our knowledge, acetone extracts of the tested lichens in our study have not been previously evaluated against MCF-7 and HepG2/C3A cell lines.

Methanolic extracts of lichens were evaluated for antioxidant potencies. Although a weak antioxidant activity has been reported for *P. furfuracea* (Odabasoglu et al., 2004; Kosanic et al., 2013), high potency of *P. furfuracea* was observed in our study (Table 2). Methanol and acetone extracts of lichens were assessed for total phenol and flavonoid contents (Table 3). Generally, acetone extracts had higher total phenol and flavonoid contents than methanol extracts. Acetone extracts of *E. prunastri* and *P. furfuracea* had the highest phenolic contents, while acetone extracts of *R. fastigiata* and *R. farinaceae* possessed the highest flavonoid contents (Table 3). While Mitrovic and co-workers (2011) showed that *E. prunastri* had low total phenol and flavonoid contents (80.73 and 27.46 mg g^{-1} dried extracts, respectively), our results exhibited higher amounts of phenolic and flavonoid capacities for *E. prunastri*. Consistent to the study of Mitrovic and co-workers (2014), total phenolic contents of *P. glauca* and *P. furfuracea* ranged from 39.75 to 63.69 and 123.09 to 131.92 mg GAE/g dry weight, respectively. On the other hand, in the same study, *P. glauca* and *P. furfuraceae* showed around 6 and 3 fold lower antioxidant value than our result, respectively (DPPH, IC_{50}: 656.98 and 95.33 μg ml^{-1}, respectively). Moreover, Ristic and co-workers (2016) showed low DPPH scavenging activity (IC_{50}: 423.51 μg ml^{-1}) and phenolic contents (33.49 mg g^{-1} of dried extract) for *R. fastigiata*, but higher phenolic content and stronger DPPH radical scavenging activity were exhibited in our study (Table 2 and 3).

Table 2. Antiproliferative and antioxidant (DPPH) activities of tested lichen species

Lichen species	IC_{50} (μg ml^{-1})		
	Antiproliferative activity		Antioxidant activity
	Cell lines		
	MCF-7	HepG2/C3A	
E. prunastri	>200	>200	398.2
P. glauca	>200	111.7±1.7	226.4
P. furfuracea	146.5±2.8	184.3±0.4	28.3
R. fastigiata	>200	167.8±0.0	111.1
R. farinacea	>200	>200	486.1

Data are presented as mean±standard error (SE). IC50: half maximal inhibitory concentration

Table 3. Total phenol and flavonoid contents of tested lichen species

Lichen species	Extract	Total phenolics mg GAE/g dried mass	Total flavonoids mg CE/g dried mass
E. prunastri	AE	213.4± 0.01	140.4±0.00
	ME	153.1±0.00	88.5±0.00
P. glauca	AE	43.1±0.01	81.6±0.00
	ME	47.2±0.00	106.9±0.00
P. furfuracea	AE	186.4±0.00	71.6±0.00
	ME	97.4±0.00	104.0±0.00
R. fastigiata	AE	134.2±0.00	373.8±0.00
	ME	39.4±0.00	94.8± 0.00
R. farinacea	AE	95.7±0.01	189.3±0.00
	ME	51.2±0.00	134.9±0.00

Data are presented as mean±standard error (SE). AE: acetone; ME: methanol; GAE: gallic acid equivalent; CE: catechol equivalent

Zones of inhibition of bacterial growth by tested lichen species are reported in Table 4. Mostly, methanol extracts of tested lichen species exhibited higher antibacterial activity than acetone extracts. All lichen extracts were found to be active against Gram-positive bacteria (*S auerus, S. epidermidis,* and *S. pyogenes*). None of the tested lichen extracts showed antibacterial activity against *S. typhimurium, P. aeruginosa,* and *E. coli* (data not shown).

Among the tested lichen species, the methanol extract of *R. fastigiata* showed the best antibacterial activity with inhibition against 6 out of 7 bacteria. Preceding studies (Cansaran et al., 2007; Şahin et al., 2015; Ristic et al., 2016) reported that acetone extract of *R. fastigiata* showed effect against *E. coli* and *S. aureus*. Here, the antibacterial activity of *R. fastigiata* against *S. epidermidis*, *S. pyogenes*, *P. vulgaris*, *K. pneumonia*, and *E. cloacae* was investigated for the first time. Both acetone and methanol extracts of *P. furfuracea* showed strong activity against Gram-positive bacteria. Former studies reported that *P. furfuracea* exhibited antibacterial activity against *E. coli, K. pneumonia, S. aureus*, and *P. vulgaris* (Turk et al., 2006; Güvenç et al., 2012; Kosanic et al., 2013). However, inhibition of *S. epidermidis* and *S. pyogenes* by *P. furfuracea* was reported for the first time in our study. It was reported that *R. farinacea* demonstrated inhibition effect against *S. aureus, S. epidermidis, E. coli*, and *P. vulgaris* (Tay et al., 2004; Karagöz et al., 2009; Şahin et al., 2015). The methanol extract of *E. prunastri* was more effective than acetone extract. However, Aslan and co-workers (2006) demonstrated weak antibacterial activity for methanol extract of *E. prunastri*. The acetone extract of *P. glauca* was found to be more active than methanol extracts against all Gram-positive bacteria. Mitrovic and co-workers (2014) showed that it was active against *S. aureus, P. aureginosa, E. coli,* and *S. typhimurium*, while we demonstrated the antibacterial activity of *P. glauca* against Gram-positive *S. aureus*. The inhibitory activity of *P. glauca* against *S. epidermidis* and *S. pyogenes* was examined for the first time in this study.

Table 4. Antibacterial activity of tested lichen species and controls

		Zone of inhibition (mm±SE)						
Lichen species	Extract	*S. auerus*	*S. epidermidis*	*S. pyogenes*	*S. marcescens*	*P. vulgaris*	*K. pneumonia*	*E. cloacae*
E. prunastri	AE	16.0±0.6^{i}	23.5±0.5f^{g}	25.0±1.3^{f}	-	10.0±0.0^{de}	-	-
	ME	16.3±0.3^{i}	24.5±0.3^{f}	27.5±0.9^{ef}	7.5±0.3^{f}	11.5±0.3^{cd}	-	-
P. glauca	AE	12.0±0.6^{k}	13.8±0.5^{h}	18.0±0.4^{g}	-	-	-	-
	ME	10.0±0.0^{l}	10.0±0.0^{i}	19.5±0.3^{g}	-	-	-	-
P. furfuracea	AE	22.3±0.5^{f}	24.5±0.3^{f}	27.0±1.3^{ef}	-	-	-	-
	ME	20.3±0.3^{g}	22.5±0.3^{g}	30.5±0.5^{de}	-	13.5±0.3^{c}	-	-
R. fastigiata	AE	19.3±0.3^{h}	26.0±0.4^{e}	29.5±0.5^{ef}	-	10.5±0.3^{d}	-	-
	ME	16.5±0.3^{i}	29.5±0.5^{d}	29.3±0.5^{ef}	-	11.3±0.3^{cd}	13.3±0.3^{b}	19.3±0.5^{e}
R. farinacea	AE	14.8±0.3^{j}	24.5±0.3^{f}	29.0±0.6^{ef}	-	7.5±0.3^{e}	-	-
	ME	15.3±0.3^{ij}	30.3±0.3^{cd}	29.8±0.3^{ef}	-	-	-	-
Ampicillin (10 mg)		39.8±0.8^{b}	31.0±0.0^{c}	48.6±2.5^{a}	14.6±0.3^{d}	27.0±1.4^{b}	8.4±1.1^{c}	27.0±0.0^{d}
Carbenicillin (100 mg)		43.8±0.8^{a}	40.0±1.4^{a}	42.8±3.3^{b}	28.0±0.0^{a}	36.8±1.9^{a}	8.0±1.4^{c}	32.0±1.4^{a}
Chloramphenicol (30 mg)		25.0±0.0^{e}	32.6±1.7^{b}	32.0±1.4^{cde}	26.8±0.8^{b}	27.0±1.4^{b}	29.2±0.8^{a}	28.8±0.5^{c}
Erythromycin (15 mg)		30.0±0.0^{d}	40.0±0.0^{a}	35.2±3.6^{cd}	11.0±0.0^{e}	13.6±0.3^{c}	13.0±0.0^{b}	9.6±0.3^{f}
Tetracycline (30 mg)		32.0±0.0^{c}	8.0±0.0^{j}	36.2±1.2^{c}	23.8±0.8^{c}	37.6±1.6^{a}	29.8±0.5^{a}	30.6±0.3^{b}
DMSO		-	-	-	-	-	-	-

Data are presented as mean diameter of inhibition zones±standard error (SE). Means with the same letter within columns are not significantly different at P>0.05. AE: acetone; ME: methanol; DMSO: dimethyl sulfoxide

3. Conclusions

The current study reported the following findings for the first time: 1) quantitative analysis of atranorin, evernic acid, and usnic acid in tested lichen species growing in Turkey; 2) antiproliferative activity of acetone extract of *P. glauca* followed by *R. farinacea* against HepG2/C3A cell line, and also activity of *P. furfuracea* against both HepG2/C3A and MCF-7 cell line; 3) the potent DPPH radical scavenging activity of *P. furfuracea* and high phenolic contents of acetone extracts of *E. prunastri* and *P. furfuracea*; 4) antibacterial activity of *P. glauca, P. furfuracea*, and *R. fastigiata* against *S. epidermidis* and *S. pyogenes,* and also antibacterial activity of *R. fastigiata* against *P. vulgaris*, *K. pneumonia*, and *E. cloacae*. Taken together, further research is required to elucidate the mechanism of action of bioactive secondary metabolites detected in lichen species. The results of our study provide promising guideline regarding the potential uses of tested lichen species as a source of anticancer, antioxidant, and antibacterial agents.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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