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ORIGINAL ARTICLE

EVALUATION OF PHARMACEUTICAL POTENTIAL AND PHYTOCHEMICAL ANALYSIS OF SELECTED TRADITIONAL LICHEN SPECIES

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Abstract

Lichens have been commonly used in traditional medicine. Biological potential (anti-proliferative, antioxidant and anti-bacterial) and chemical content of five lichen species (*E. divaricata, L. vulpina, L. pulmonaria, R. fraxinea* and *U. florida*) were assessed in relation to traditional knowledge. Consistent with folkloric usage, the strongest anti-proliferative activity was observed with *L. pulmonaria* against human hepatocellular carcinoma (HepG2/C3A) cell line. *L. pulmonaria* also showed the highest antioxidant capacity. While *E. divaricata* had the most phenolic content, the highest flavonoid content was determined in *L. pulmonaria*. All lichen extracts showed the best antibacterial activity against Gram-positive bacteria and only *R. fraxinea* had a broad spectrum of antibacterial activity. HPLC-DAD analysis revealed that *L. pulmonaria* and *U. florida* were the best sources of stictic acid and usnic acid, respectively. Traditional usages of tested lichens were justified with this study and nutraceutical potentials of them were revealed.

Rezumat

Studiul prezintă potențialul biologic (antiproliferativ, antioxidant și antibacterian) și conținutul chimic a cinci specii de licheni (*E. divaricata, L. vulpina, L. pulmonaria, R. fraxinea* și *U. florida*) care au fost evaluate în raport cu utilizările tradiționale. Cea mai intensă activitate antiproliferativă a fost observată pentru *L. pulmonaria* asupra liniei celulare de carcinom hepatocelular uman (HepG2/C3A). *L. pulmonaria* a prezentat, de asemenea, cea mai pronunțată capacitate antioxidantă. În timp ce *E. divaricata* a prezentat cel mai mare conținut fenolic, cel mai mare conținut de flavonoide a fost determinat în *L. pulmonaria*. Toate extractele de lichen au prezentat cea mai bună activitate antibacteriană împotriva bacteriilor Gram-pozitive și doar *R. fraxinea* a avut un spectru larg de activitate antibacteriană. Analiza HPLC-DAD a arătat că *L. pulmonaria* și *U. florida* reprezintă cele mai bune surse de acid stictic și, respectiv, acid usnic. Utilizările tradiționale ale lichenilor testați au fost justificate prin acest studiu și au fost dezvăluite potențialele nutraceutice ale acestora.

Keywords: **a**ntibacterial, antioxidant, anti-proliferative, HPLC, lichen

Introduction

Lichens are important traditional medicines for treating skin, respiratory, digestive and urinary problems. Lichens have been widely used as anti-inflammatory, styptic and vulnerary in many different cultures including USA, Europe, India and China since ancient times [6]. Table I provides the details on the traditional usage of each lichen species tested in our research.

In the light of ethnobotanical information, the present study aimed to assess the antioxidant capacity, anti-bacterial and antiproliferative potentials of five lichen species: *Evernia divaricata* (L.) Ach., *Letharia vulpina* (L.) Hue, *Lobaria pulmonaria* L. (Hoffm.), *Ramalina fraxinea* (L.) Ach. and *Usnea florida* (L.) Weber ex. Wigg.; and to elucidate their chemical contents by HPLC-DAD in order to provide possible therapeutic agents.

Materials and Methods

Lichen material and extraction

Five different lichen species (*E. divaricata, L. vulpina, L. pulmonaria, R. fraxinea* and *U. florida*) were collected from several provinces (Lake Abant province, Aladağlar province and Lake Seben province) of Bolu, Turkey. Extraction procedures were performed according to the method described by Tas *et al.* [15].

Anti-proliferative activity

Anti-proliferative activities of acetone extracts of the lichens against human breast adenocarcinoma (MCF-7)

and human hepatocellular carcinoma (HepG2/C3A) cell lines were determined by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay according to the procedure reported by Tas *et al.* [15].

Free radical scavenging activity

Free radical scavenging activity of the methanolic extracts of lichen species was evaluated by using 2,2-diphenyl-1-picrylhydrazil (DPPH•, Sigma®) at 517 nm according to the procedure described by Tas *et al*. [15].

Determination of total phenolic and flavonoid content

Total phenolic contents by using a Folin-Ciocâlteu phenolic reagent and total flavonoids by using aluminium chloride ($AlCl_3$) colorimetric assay were determined in the acetone and methanolic extracts according to the method described by Tas *et al.* [15].

Antibacterial bioassay

For the antibacterial activity screening, the disc diffusion assay was performed against ten pathogenic bacteria according to the method described by Tas *et al*. [15].

High-Performance Liquid Chromatography (HPLC) analysis

Five different lichen metabolites were used as reference standards consisting of usnic acid (Sigma®), evernic acid (Sigma®), stictic acid (Chromadex®), atranorin (Chromadex®) and fumarprotocetraric acid (BOC sciences®). HPLC analysis was conducted according to the method described by Tas *et al.* [15].

Statistical analysis

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

Results and Discussion

Five lichen species used in traditional medicine (Table I) were evaluated for their anti-proliferative (Table II), antioxidant (Table II and III) and anti-bacterial potentials (Table IV) and phytochemical content (Table V).

Table I

Information about tested lichen species and their usage in traditional medicine around the world [6]

Lichen Species	Family	Main area of use	Traditional uses
E. divaricata	*Parmeliaceae*	China	Used for coughs, pneumonia, hot flashes due to pulmonary tuberculosis, hepatitis, headaches, infection due to trauma, inflammation of the breasts and snakebites.
L. pulmonaria	*Lobariaceae*	Europe, India, Afghanistan, China	Mainly used in lung diseases including tuberculosis, asthma, coughs, spitting blood, but also for liver diseases, as an appetite stimulant, for diarrhoea, for heavy menstrual flow, for oedema due to kidney inflammation, local swelling, reducing inflammation, relieving pain, severe itching of skin and to stop bleeding.
L. vulpina	*Parmeliaceae*	Sweden, Canada, USA	Used for wolf poison. Infusion of the lichen and bone marrow for stomach disorders like ulcers. Applied as a poultice for open sores, boils, bruises, swellings, arthritis and eye problems.
Ramalina spp.	*Ramalinaceae*	Greece, Nepal, India	Applied as a poultice, it stops bleeding, relieves inflammation and cures lichen (the skin disease). Mixed with honey it cures jaundice and smeared on the mouth and tongue, it relieves colds and congestion.
U. florida	*Parmeliaceae*	China, Europe, Chile	Used for aching in sinews and bones, stopping bleeding or infection from external injuries, skin diseases, diarrhoea, painful urination, colds, coughs, tuberculosis of lungs or neck, heart palpitations and oedema.

HepG2/C3A was more vulnerable to lichen extracts. *E. divaricata* was the only lichen that showed anti-proliferative effect against MCF-7 cell line (112.9 µg/mL). The highest activity was observed for *L. pulmonaria* (IC_{50}: 90.2 µg/mL), followed by *E. divaricata* (135.5 µg/mL) against HepG2/C3A cell line (Table II).

Crawford [6] reported that *E. divaricata* has been used for the treatment of inflammation of breasts in traditional medicine. Recently, it has been shown that inflammation is involved in the development and progression of cancer [16]. Consistent with traditional usage, we reported that *E. divaricata* suppressed the growth of breast MCF-7 and liver HepG2/C3A cancer cell lines (Table II). Treatment with vulpinic acid, the main compound of *L. vulpina*, significantly reduced cell viability in lung adenocarcinoma cell lines (A549, NCI-H1264, NCI-H1299 and Calu-6), pancreatic ductal adenocarcinoma cell lines (PANC-1 and MIA PaCa-2) and hepatocellular carcinoma cell line (HepG2) [8]. Here, we reported the effect of *L. vulpina* against HepG2/C3A and MCF-7 cell lines (Table II). Anti-cancer activity of *L. pulmonaria* was performed against human lung (A549 and H1299) and breast (MCF-7 and MDA-MB-231) cancer cell lines by Ozturk *et al.* [11]. It was reported that *L. pulmonaria* had been used in the case of liver diseases in traditional medicine [6]. Consistently, we revealed that the strongest cytotoxicity was observed by *L. pulmonaria* (IC_{50}: 90.2 µg/mL) against HepG2/C3A cell line. Ristic *et al.* [12] reported that *R. fraxinea* treatment exhibited anti-cancer activity against human epithelial (Hela), lung

(A549) and colon (LS174) cancer cells. To our knowledge, this is the first report that exhibits the anti-proliferative effect of *R. fraxinea* against HepG2/C3A (IC_{50}: 148.3 µg/mL) and MCF-7 (IC_{50}: > 200 µg/mL) cell lines (Table II).

Table II

Anti-proliferative and antioxidant potential of tested lichen species

Lichen species	IC_{50} (µg/mL)		
	MCF-7	HepG2/C3A	Antioxidant (DPPH)
E. divaricata	112.9 ± 0.5	135.5 ± 0.0	672.02
L. pulmonaria	> 200	90.2 ± 1.2	39.05
L. vulpina	> 200	175.7 ± 1.4	74.16
R. fraxinea	> 200	148.3 ± 2.2	130.2
U. florida	> 200	181.9 ± 2.1	1276.03

Data were presented as a mean number ± standard error (SE). IC_{50}: The half maximal inhibitory concentration.

The scavenging activity of antioxidant substances is evaluated using DPPH radical as a substrate. Phenolics and flavonoids are significantly effective scavengers of free radicals [4, 5, 7]. *L. pulmonaria* exhibited the highest DPPH radical scavenging activity (IC_{50}: 39.05 µg/mL), while the remaining species resulted in the inhibition of DPPH in the ranged from 74.16 to 1276.03 µg/mL (Table II). Acetone extract of *E. divaricata* had the highest phenolic content (215.7 GAE/g dry weight), while *R. fraxinea* and *L. pulmonaria* possessed the highest flavonoid contents (356.0 and 207.1 mg CE/g dry weight, respectively) (Table III). Consistent with our antioxidant capacity (IC_{50}: 39.05 µg/mL) and total phenolic content (82.4 mg GAE/g) results for *L. pulmonaria*, Odabasoglu *et al*. [10] reported that methanol extract of *L. pulmonaria* showed potent anti-oxidant activities and had the highest total phenolic contents (87.9 mg GAE/g). Therapeutic potential of *L. pulmonaria* in folk medicine in the treatment of lung, liver and kidney diseases may come from high antioxidant capacity of this lichen.

E. divaricata and *U. florida* showed lower DPPH activity (Table II). Similar to our results, Aslan *et al*. [3] reported that methanol extract of *E. divaricata* did not exhibit antioxidant activity in DPPH assay. Consistent with previous report [10], the correlation was not observed between antioxidant activity and total phenolic content for *E. divaricata*. Methanol extract of *R. fraxinea* exhibited stronger antioxidant potential with IC_{50} value as 130 µg/mL in our study. Şahin *et al*. [13] reported that total phenolic contents of methanol and acetone extract of *R. fraxinea* were 103.3 mg GAE/g and 70 mg GAE/g, respectively. However, while methanol and acetone extracts of *R. fraxinea* had lower total phenolic content (36.4 and 26 mg GAE/g, respectively), exhibited high amount of flavonoid content (94.3 and 356 CE/g, respectively) in our study (Table III). *L. vulpina* showed strong antioxidant capacity (IC_{50} value as 74.16 µg/mL) in our study. Similarly, Anar *et al*. [1] revealed strong antioxidative and antigenotoxic effects of *L. vulpina* in human lymphocytes *in vitro*.

Table III

Total phenol and flavonoid capacity of tested lichen species

Lichen species	Treatments	Total Phenolics mg GAE/g dried mass	Total Flavonoids mg CE/g dried mass
E. divaricata	AE	215.7 ± 0.00	116.0 ± 0.00
	ME	118.0 ± 0.00	79.1 ± 0.00
L. pulmonaria	AE	122.7 ± 0.00	207.1 ± 0.00
	ME	82.4 ± 0.00	114.2 ± 0.00
L. vulpina	AE	53.8 ± 0.00	164.9 ± 0.00
	ME	27.5 ± 0.00	88.5 ± 0.00
R. fraxinea	AE	26.0 ± 0.00	356.0 ± 0.00
	ME	36.4 ± 0.00	94.3 ± 0.001
U. florida	AE	113.8 ± 0.00	140.4 ± 0.00
	ME	37.5 ± 0.00	116.0 ± 0.00

Data were presented as a mean number ± standard error (SE). AE: acetone, ME: methanol, GAE: gallic acid equivalent, CE: catechol equivalent.

The diameters of inhibition zone for acetone and methanol extractions of the 5 lichen species against 10 different bacterial strains were reported in Table IV. Generally, Gram-positive bacteria (*S auerus, S. epidermidis* and *S. pyogenes*) in our experiment were more susceptible to the inhibitory effects of the lichens than Gram-negative bacterial strains (Table IV).

Among all lichens tested, acetone and methanol extracts of *L. vulpina* exhibited the strongest anti-bacterial activity against *S. aureus* and *S. epidermidis* that were higher than some of the reference anti-biotics. *L. vulpina* also showed antibacterial activity against *P. vulgaris*. This lichen species has been recorded to be used in traditional medicine for open sores, boils, bruises, swellings, arthritis, and eye problems

[6]. Folkloric therapeutic usage of this lichen may be due to its antibacterial activity against *S. aureus*, *S. epidermidis* and *P. vulgaris*. Both acetone and methanolic extracts of *E. divaricata* showed relatively strong antibacterial activity against Gram positive bacterial strains only. This may explain the reason for usage to treat coughs, pneumonia, and inflammation of the breasts. Among the tested lichens, only *R. fraxinea* had a broad spectrum of antibacterial activity by suppressing both Gram-positive and Gram-negative bacteria. It demonstrated the best antibacterial activity against Gram-negative bacteria except *K. pneumonia* and *E. coli*. The usage of *Ramalina* spp. as curative purpose in the common cold, skin diseases and inflammations [6] explains the broad-spectrum antibacterial activity of *R. fraxinea*. Both acetone and methanolic extract of *L. pulmonaria* were determined to be active against Gram-positive bacteria. The acetone extract of *L. pulmonaria* showed the highest antibacterial activity against *S. pyogenes* which was higher than some of the reference antibiotics. *L. pulmonaria* has become famous for centuries in the treatment of lung diseases [6]. Strong susceptibility of Gram-positive bacteria against *L. pulmonaria* extracts may explain and justify this traditional usage. Both extracts of *U. florida* suppressed the growth of Gram-positive bacteria, verifying the folkloric usage for skin diseases, urinary tract infections, common cold and coughs.

Table IV

Antibacterial potential of the tested lichens and controls

Treatments		Zone of inhibition (mm ± SE)									
		S. auerus	*S. epidermidis*	*S. pyogenes*	*S. marcescens*	*S. typhimurium*	*P. aeruginosa*	*P. vulgaris*	*K. pneumonia*	*E. cloacae*	*E. coli*
E. divaricata	AE	20.0 ± 0.0^{f}	31.0 ± 0.6^{bc}	32.0 ± 0.0^{cde}	-	-	-	-	-	-	-
	ME	20.5 ± 0.3^{f}	30.3 ± 0.3^{c}	35.0 ± 0.0^{cd}	-	-	-	-	-	-	-
L. vulpina	AE	31.5 ± 0.5^{c}	30.0 ± 0.9^{cd}	-	-	-	-	10.5 ± 0.5^{de}	-	7.5 ± 0.3^{g}	-
	ME	30.8 ± 0.8^{cd}	30.5 ± 0.5^{c}	-	-	-	-	10.0 ± 0.0^{de}	-	-	-
L. pulmonaria	AE	14.8 ± 0.3^{h}	21.8 ± 0.3^{f}	36.5 ± 0.5^{c}	-	-	-	8.5 ± 0.3^{e}	-	-	-
	ME	9.3 ± 0.3^{i}	11.3 ± 0.3^{g}	17.0 ± 0.6^{g}	-	-	-	7.8 ± 0.5^{e}	-	-	-
R. fraxinea	AE	15.0 ± 0.4^{h}	30.5 ± 0.5^{c}	27.0 ± 1.3^{ef}	-	-	-	-	-	-	-
	ME	16.3 ± 0.3^{g}	28.3 ± 0.6^{d}	30.5 ± 1.3^{def}	9.5 ± 0.3^{f}	14.8 ± 0.3^{c}	18.8 ± 0.8^{a}	12.3 ± 0.3^{cd}	-	23.0 ± 0.0^{e}	-
U. florida	AE	13.8 ± 0.3^{h}	24.8 ± 1.4^{e}	27.0 ± 0.6^{ef}	-	-	-	-	-	-	-
	ME	14.5 ± 0.3^{h}	31.5 ± 0.5^{bc}	25.5 ± 0.5^{f}	-	-	-	-	-	-	-
Ampicillin (10 mg)		39.8 ± 0.8^{b}	31.0 ± 0.0^{bc}	48.6 ± 2.5^{a}	14.6 ± 0.3^{d}	27.4 ± 0.3^{a}	-	27.0 ± 1.4^{b}	8.4 ± 1.1^{c}	27.0 ± 0.0^{d}	21.2 ± 0.5^{c}
Carbenicillin (100 mg)		43.8 ± 0.8^{a}	40.0 ± 1.4^{a}	42.8 ± 3.3^{b}	28.0 ± 0.0^{a}	25.2 ± 2.2^{b}	10.0 ± 0.0^{c}	36.8 ± 1.9^{a}	8.0 ± 1.4^{c}	32.0 ± 1.4^{a}	24.4 ± 0.3^{b}
Chloramphenicol (30 mg)		25.0 ± 0.0^{e}	32.6 ± 1.7^{b}	32.0 ± 1.4^{cde}	26.8 ± 0.8^{b}	28.8 ± 0.8^{a}	9.0 ± 0.0^{d}	27.0 ± 1.4^{b}	29.2 ± 0.8^{a}	28.8 ± 0.5^{c}	28.2 ± 0.5^{a}
Erythromycin (15 mg)		30.0 ± 0.0^{d}	40.0 ± 0.0^{a}	35.2 ± 3.6^{cd}	11.0 ± 0.0^{e}	10.8 ± 0.5^{d}	-	13.6 ± 0.3^{c}	13.0 ± 0.0^{b}	9.6 ± 0.3^{f}	12.0 ± 0.0^{d}
Tetracycline (30 mg)		32.0 ± 0.0^{c}	8.0 ± 0.0^{h}	36.2 ± 1.2^{c}	23.8 ± 0.8^{c}	25.2 ± 0.5^{b}	16.0 ± 0.0^{b}	37.6 ± 1.6^{a}	29.8 ± 0.5^{a}	30.6 ± 0.3^{b}	28.8 ± 0.8^{a}
DMSO		-	-	-	-	-	-	-	-	-	-

Data were presented as a 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.

We reported that *S. epidermidis*, *E. cloacae* and *P. vulgaris* were inhibited by *L. vulpina* for the first time. Although Shrestha *et al*. [14] reported that *L. pulmonaria* had no inhibitory activity against *S. aureus*, here we showed that it has antibacterial effect against *S. aureus* with both extracts. Moreover, we revealed the antibacterial activity of *L. pulmonaria* against *S. epidermidis*, *S. pyogenes* and *P. vulgaris*. Preceding studies [12, 13] reported the inhibition effect of *R. fraxinea* against *E. coli* and *S. aureus*. This is the first report that the methanol extract of *R. fraxinea* showed antibacterial activity against *S. marcescens*, *S.*

pyogenes, *S. typhimurium*, *P. aureginosa*, *P. vulgaris* and *E. cloaca*. In addition, we reported the growth inhibition of *S. pyogenes* and *S. epidermidis* by *U. florida* for the first time.

Quantitative analyses of lichen acids were achieved using HPLC-DAD system (Table V). Both acetone and methanolic extracts of *E. divaricata* contained usnic acid (49.10 and 5.71 mg/g dry extract, respectively). Atranorin was only detected in the acetone extract of *L. vulpina* (8.45 mg/g dry extract). High amount of stictic acid was found in both acetone and methanol extracts of *L. pulmonaria* (243.3 and 117.80 mg/g dry extract, respectively). Potent antioxidant and anti-proliferative activity of *L. pulmonaria* may depend on its high stictic acid content. Acetone extract of *U. florida* contained the most considerable amount of usnic acid (440.98 mg/g dry extract) as expected. Both *U. florida* and *E. divaricata* included valuable amount of usnic acid. However, *E. divaricata* exhibited better antibacterial and anti-proliferative activity. The results suggest that main compound of *E. divaricata*, divaricatic acid [18], has promising bioactivity or synergetic potential together with usnic acid. On the other hand, *R. fraxinea* showed poor usnic acid content in both extracts (Table V).

Table V

Quantitative analysis of tested lichen species by HPLC-DAD

Lichens Species		Standard Compounds (mg/g dry extract)				
		Atranorin	Evernic acid	Fumarprotocetraric acid	Stictic acid	Usnic acid
E. divaricata	AE	-	-	-	-	49.10 ± 0.02
	ME	-	-	-	-	50.71 ± 0.86
L. vulpina	AE	8.45 ± 0.17	-	-	-	-
	ME	-	-	-	-	-
L. pulmonaria	AE	-	-	-	243.30 ± 0.60	-
	ME	-	-	-	117.80 ± 0.36	-
R. fraxinea	AE	-	-	-	-	9.24 ± 0.04
	ME	-	-	-	-	16.21 ± 0.12
U. florida	AE	-	-	-	-	440.98 ± 0.25
	ME	-	-	-	-	99.41 ± 0.26

Data were presented as a mean number of lichen acid amount ± standard error (SE). AE: acetone, ME: methanol.

Lichens are exposed to stressful factors in a wide range of natural environments such as low temperatures, prolonged darkness, drought, and continuous light [9]. Aoussar *et al*. [2] showed that the harvesting period of the lichens had a significant impact on phenolic content and antioxidant capacity. In another report, Vatne *et al*. [17] reported that biotic and abiotic stress conditions affected the carbon-based chemical contents of lichen species. For these reasons, the chemical composition of lichens may show differences among the reported researches depending on their harvesting region, elevation, season and others.

Conclusions

Validation of traditional usage of medicinal plants through scientific studies is very valuable for mankind. This study provided a connection and scientific evidence from a traditional remedy to modern therapy for tested lichen species. Our study provided the first evidence of the strong anti-proliferative activity of *L. pulmonaria* against liver cancer cell line (HepG2/C3A) supported by the folkloric usage in the treatment of liver diseases. Moreover, traditional knowledge of *E. divaricata* as a remedy for breast inflammation can be justified with potent anti-proliferative activity of this lichen species against breast cancer cell line (MCF-7) for the first time. Further studies should focus on the biological activity-guided isolation of the active constituents for potent lichen species.

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Conflict of interest

The authors declare no conflict of interest.

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