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Comparative *in vitro* analysis of the biological potential of *Usnea florida* (L.) Weber ex F.H. Wigg., *Usnea intermedia* (A. Massal.) Jatta, and *Usnea lapponica* vain and quantification of usnic acid

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The present study focused on the antioxidant, cytotoxic, antimicrobial, and DNA-protective effects of *Usnea lapponica, Usnea intermedia*, *and Usnea florida*, which are distributed in Turkey. The methanol extracts of these three lichen species were subjected to high-performance liquid chromatography (HPLC) analysis to quantify the amount of usnic acid and the highest amount was found in *U. lapponica* (3345.9±92.18 mg/$g_{extract}$). Furthermore, the total phenolic and flavonoid contents were analyzed. Antioxidant capacity was evaluated by different chemical assays, together with antimicrobial, cytotoxic, and DNA-protective properties.The results of the antioxidant assays showed that *U. lapponica* exhibited the highest DPPH radical scavenging activity, whereas the extract that scavenged ABTS radicals more potently was identified as *U. intermedia*. The disc diffusion method was used to investigate the antimicrobial activity, and the strongest effect was observed in *U. intermedia* methanol extract with a zone value of 13 mm on *Micrococcus luteus.* In cytotoxicity experiments, *U. florida* extract (250 µg/mL; 36.03% viability) exhibited a high antiproliferative effect on A549 cells and *U. intermedia* (62.5 µg/mL; 23.65% viability) on MDA-MB-231 cells. DNA-protective effects were investigated using pBR322 plasmid DNA, and all the studied species were found to have DNA-protective effects. These results suggest that *Usnea* species may be potential candidates for the development of new phytopharmaceuticals and functional ingredients.

Keywords: Antimicrobial, Antioxidant, Cytotoxicity, DNA cleavage, *Usnea*

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Lichens are symbiotic organisms composed of fungi, algae, and/or cyanobacteria[1], and approximately 7% of Earth's vegetation is dominated by lichen species. Lichens synthesize various metabolites and the number of known lichen metabolites has reached 1050, and lichens are used for medical and industrial purposes[2]. The genus *Usnea* Dill. ex Adans. (Parmeliaceae) were also included in medicinal lichens. Lichens belonging to the genus *Usnea* are traditionally used for medicinal purposes in various cultures around the world. Especially *Usnea florida* and *Usnea intermedia* are mentioned in the literature for their traditional uses. *U. florida* is used for pain in muscles and bones, external injuries, skin diseases, painful urination, and cough in China. It is also used in the medication of diseases such as lung or neck tuberculosis, heart, palpitations, and bleeding or infection caused by edema by boiling it in the form of a drink or by applying lichen powder to the diseased area[3]. It is used in Europe for colds and coughs and in Chile for diarrhea[4,5]. *U. intermedia* has been internally adopted in Indonesia for treating gout, the common cold, and as a carminative[6].

The genus *Usnea* is considered one of the most taxonomically difficult species of macrolichens[7]. It comprises more than 500 taxa in the world[8]. *Usnea* can be easily recognized in the field by greenish-yellow thallus hanging from the tree, radial symmetry, and central cartilage axis[9]. Some members of the *Usnea* genus are edible and utilized in traditional meals and treatments in both Eastern and Western cultures[10].

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Antioxidants, both natural and synthetic, can help minimize oxidative stress and biomolecule damage. The ability of antioxidants to eliminate free radicals facilitates this process. Free radical species can cause a wide range of diseases, including cancer, inflammation, hypertension, and cardiovascular issues. Lichens have powerful antioxidant properties thanks to their extraordinary variety of natural compounds, including usnic acid, one of the most popular lichen phenolics. The most prevalent metabolite in the genus *Usnea* is usnic acid[11]. Studies have investigated the anti-tuberculosis, antibacterial, antiprotozoal, antimycotic, antiviral, antiulcer, antiproliferative, and anti-inflammatory, activities of this secondary metabolite[8,12]. Furthermore, the preventive properties of usnic acid against DNA damage and its potent UV-filtering agent have been clarified[13,14]. In recent studies, it has been established that *Usnea* lichen extract commonly comprises compounds capable of inducing cytotoxic effects. The antimicrobial activities and cholinesterase inhibition properties of compounds isolated from certain species have been assessed. Moreover, the high antioxidant activity of usnic acid isolated from *Usnea* sp. lichen has been underscored, unveiling the potential cytotoxic effects of lichens within the *Usnea* genus[15-19]. The present study was performed to investigate the antioxidant, cytotoxic, antimicrobial, and DNA-protective effects of methanol extracts derived from *Usnea lapponica* Vain, *Usnea intermedia* (A. Massal.) Jatta, and *Usnea florida* (L.) Weber ex F.H. Wigg. species found in Turkey. The amount of usnic acid in the three lichen species was determined by HPLC. Although there are many articles on *Usnea* species, most have focused on its antimicrobial activity. In this study, the potential of these species was revealed by evaluating different biological activities from a broad perspective, and the biological activities of *U. lapponica* were documented for the first time. This research sought to offer more information on the industrial use of *Usnea* species and the development of new products from these species such as functional foods, herbal medicines, and natural antioxidant sources.

Materials and Methods

Lichen materials

During field expeditions in 2015 and 2019, lichen specimens were systematically gathered from three distinct geographical locations. The specimens were carefully conserved in the herbarium of lichens at Yozgat Bozok University, and their identification was conducted using taxonomic keys, relying on morphological and anatomical characteristics[20,21].

The locality information and herbarium numbers of Usnea species

U. lapponica: Turkey, Kastamonu, Ilgaz Mountain National Park, *Abies nordmanniana* subsp. forest, 41°04'419" N, 33°43'331" E, 1730 m., 07/07/2015, [MK001]. *U. intermedia*: Turkey, Kastamonu, Devrekani, west of Kanlıabant, *Pinusnigra* Arnlond and *Quercus* L. communities, 41°32'433" N, 33°46'558" E1045 m., 08/07/2015, [MK002]. *U. florida*: Turkey, Yozgat, Akdağmadeni, on the Yukarıyahyasaray road, *Pinus sylvestris* L. forest, 39°36'38" N, 35°50'56" E,1759 m., 16/10/2019, [MK003].

Preparation of the extracts

Pulverized dried samples *of U. lapponica* (3.65 g), *U. intermedia* (3.40 g), and *U. florida* (14.67 g) thalli materials were extracted three times for 24 h with 80% methanol (MeOH). Extracts were evaporated to dryness under lower pressure at 37°C. After evaporation, the remaining extracts were frozen and lyophilized.

Quantification of total phenolic and flavonoid concentrations in the extracts

The total phenolic and flavonoid contents were measured in terms of gallic acid equivalents (GAE) and catechin (CA), respectively. The quantification of total phenolic content in the extracts was carried out using the Folin-Ciocalteu assay[22], while the determination of total flavonoid content was conducted through a colorimetric aluminum chloride method[23,24].

Quantification of usnic acid levels using HPLC

Chromatographic analysis was conducted using a Shimadzu LC-20AT system with a Photodiode-Array (PDA) Detector. Separations were performed on a reverse-phase Mediterranean-C18 analytical column (250 x 4.6 mm i.d., 5 µm particle size) with a flow rate of 0.5 mL/min at 22°C. The elution solvent mixture, comprising MeOH/H_2O/H_3PO_4 (75:25:0.9, v/v/v), was employed. The usnic acid content in the samples was verified by comparing the retention time and UV spectra with those of the standard usnic acid. Standard and sample solutions were injected in triplicate for a comprehensive analysis.

Determination of antioxidant capacity

The DPPH$^{\bullet}$ scavenging activity of the extracts was evaluated according to the method outlined by Gyamfi *et al.*[25]. The absorbance of the samples was measured at 517 nm after a 30 min incubation in darkness at the optimal temperature.

The ABTS$^{+\bullet}$scavenging activity was assessed following the protocol outlined by Re *et al*.[22]. The reaction kinetics were monitored at 734 nm over 30 min at 1 min intervals. Trolox (TEAC) was used to determine the percentage of inhibition concerning concentration.

Cytotoxic Effects on A549 and MDA-MB-231 cell lines

The cytotoxic potential of the extracts was assessed in A549 (ATCC CCL-185, Human Lung Cancer Cell Line) and MDA-MB-231 (ATCC CCL-222, Human Breast Cancer Cell Line) cells using the MTT colorimetric technique. MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle's Medium, while A549 cells were cultured in Roswell Park Memorial Institute Medium. Cells were seeded in 96-well plates at a density of 1 x10^4 per well, and various concentrations of extracts (3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL) were added to each well. After 24 h of incubation, the wells were aspirated, and 100 µL of MTT solution (prepared in PBS at 0.5 mg/mL) was introduced. Following a three-hour incubation, the wells were emptied, and 100 µL of DMSO was applied. Subsequently, the absorbance was measured at a wavelength of 540 nm using ELISA (Bio-Rad Laboratories Inc., USA). The experiments were carried out in triplicate, and the results are reported as mean ± SD[26].

Antimicrobial efficacy assessment

The antimicrobial activity was evaluated against seven microbial organisms using the agar disc diffusion method[27]. The indicator bacteria employed in the assay comprised three Gram-negative bacteria (*Proteus mirabilis* ATCC 25933, *Escherichia coli* ATCC 25922, and *Enterobacter aerogenes* ATCC 13048) and three Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 10240, and *Staphylococcus aureus* ATCC 25923). The fungal isolate *Candida albicans* was procured from the culture stock repository maintained at the Faculty of Pharmacy, Erciyes University. All microorganisms were cultivated overnight at 37°C on agar plates and subsequently utilized as the inoculum. The turbidity of the suspensions was standardized to a 0.5 McFarland standard, equivalent to approximately 1.5 x108 colony-forming units per milliliter (cfu/mL). Subsequently, 100 µL of the microbial dilution was evenly distributed over the entire surface of the agar plates. Filter paper discs (6 mm in diameter, Oxoid) impregnated with 20 µL of the extracts were then positioned on the agar surfaces. The agar plates were then incubated at 37°C for 18-24 h and at 28ºC for 48-72 h for bacteria and fungi, respectively. Ampicillin (amp) and nystatin (ns) were used as reference antibiotics for bacteria and fungi, respectively. A negative control was established using paper discs impregnated with 20 µL of DMSO. The experiments were performed in triplicate, and the antimicrobial activities were assessed based on the mean diameter of the inhibition zone produced by the *Usnea* extracts.

DNA cleavage

DNA damage inhibition efficiency was determined using pBR322 plasmid DNA as described by Korkmaz *et al.,* 2018[28]. PBR322 was damaged in the presence of *Usnea* extract after exposure to UV and H_2O_2, and imaging was performed. Test samples were studied with 1.5% agarose gel at 80 V for 90 min. The agarose gel was stained with ethidium bromide and captured using a Bio-Rad Molecular Imager ChemiDoc XRS system (BioRad).

Statistical analysis of experimental data

Assessment of Variance Homogeneity via the Levene Test, Utilization of One-Way Analysis of Variance for Multigroup Comparison, Implementation of Dunnett and Tukey Tests for Multiple Comparison at the $p<0.05$ Level, and Calculation of IC50 Values through Nonlinear Regression Curves (Sigma Plot 2001, version 7.0, SPSS Inc., Chicago, IL, USA).

Results

Total phenolic and flavonoid contents

The extracts' total phenol and total flavonoid contents were determined with spectrophotometric methods, and the results are given in Table 1. According to the results, the highest phenol content among all the extracts was 87.62±4.37 $mg_{GAE}/g_{extract}$ in *U. lapponica*. The highest total flavonoid amount was determined as 23.06±3.34 $mg_{CA}/_{extract}$ in *U. florida*.

Quantification of usnic acid by HPLC

Analysis by HPLC was applied to determine the amount of usnic acid in the extracts (Fig. 1). *U. lapponica* was recorded as containing the highest

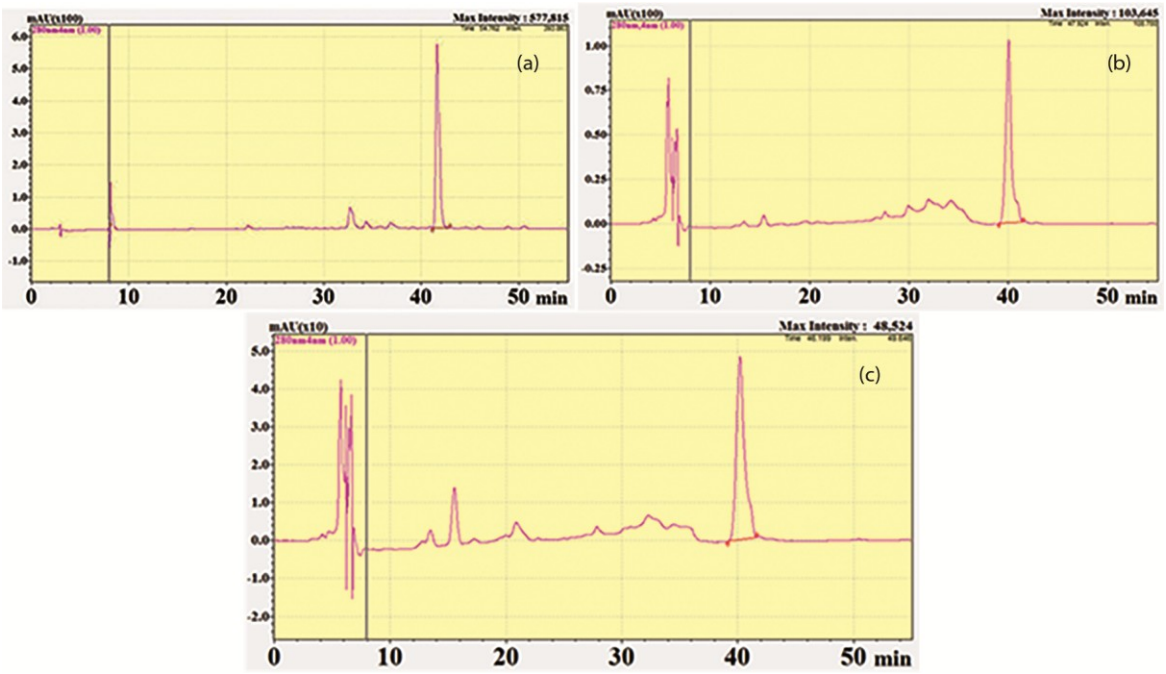

Fig. 1 — Chromatogram of (a) *U. lapponica*, (b) *U. intermedia,* and (c) *U. florida* extract

level of usnic acid (3345.9±92.18 mg/g) compared to other extracts. The usnic acid amount in the *U. intermedia* extract was found to be 1337.57±159.76 mg/g. On the other hand, with 842.95±56.56 mg/g of usnic acid, *U. florida* was likewise the extract with the lowest usnic acidamount (Table 1).

DPPH$^{\bullet}$ scavenging activity

The antiradical effects of the samples were measured using the DPPH radical. Percentage inhibition values were computed after extracts were examined at concentrations of 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, and 4 mg/mL (Table 2). *U. florida* was the most effective extract at all measured concentrations. *U. lapponica* was found more effective than the other extracts at 0.5 mg/mL and 1 mg/mL concentrations. At all tested concentrations, none of the extracts performed as well as the positive control BHT though.

ABTS$^{+\bullet}$ scavenging activity

Since there was no significant difference in ABTS$^{\bullet+}$ scavenging activity at concentrations above 2 mg/mL and below 0.5 mg/mL, the range of 0.5, and 2 mg/mL concentrations was used in this experiment. The most active species was determined as *U. intermedia* (Table 3). In addition, it was determined that all extracts of the *Usnea* species had the same statistical significance with BHT at 2 mg/mL concentration.

Table 1 — Total phenol and total flavonoid and usnic acid contents of *Usnea* species

Exracts	Yield	Total phenol [mg_{GAE}/g_{exract}]	Total flavonoid [mg_{CA}/g_{exract}]	Usnic acid (mg/g)
U. lapponica	24.2%	87.62±4.37	17.74±0.41	3345.9±92.18
U. intermedia	17.8%	73.74±1.62	17.95±3.23	1337.57±159.76
U. florida	21.6%	85.15±2.12	23.06±3.34	842.95±56.56

Data are expressed as mean ± standard error (n=3)

Cytotoxicity on A549 and MDA-MB-231 cells

The cytotoxic potential of the extracts derived from *Usnea* species was evaluated on A549 and MDA-MB-231 cancer cell lines using the MTT method. Results are given as a percentage (%), cell control group viability is assumed as 100%, and other groups are calculated.To calculate the IC_{50} values of the extracts, 3.906 µg/mL, 7.8125µg/mL, 15.625 µg/mL, 31.25 µg/mL, 125µg/mL, 250 µg/mL, 500 µg/mL, and 1000 µg/mL concentrations were prepared by serial dilution (Fig. 2 and Fig. 3).

In the A549 cell line, only the *U. florida* extract showed a cytotoxic effect (36.03%) with viability below 50% at 250 µg/mL. At 125 µg/mL concentration, none of the lichen species could decrease the viability below 50%. At 1000 µg/mL, *U. intermedia* (23.99%), *U. lapponia* (25%), and *U. florida* (25.68%) extracts were found to be significantly cytotoxic to A549 cells (Fig. 2). The IC_{50} values of *U. lapponica, U. florida, and U. intermedia*

Table 2 — DPPH$^{\bullet}$radical scavenging activity of *Usnea* species

	% Inhibition					
	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL	0.25 mg/mL	0.125 mg/mL
U. lapponica	66.32±3.11**	61.37±4.71*	61.05±3.59*	52.10±3.16	40.71±5.68	26.90±8.22
U. intermedia	62.46±5.04*	57.87±3.42*	55.99±3.46	42.91±7.41	37.71±5,67	23.60±7.21
U. florida	70.84±2.48**	64.04±4.56*	59.91±1.15*	49.60±4.76	44.52±4.6	39.4±5.38
BHT	85.4±3.02***	82.3±3.01**	78.8±3.8**	66.0±4.5**	55.08±3.2	40.59±5.4

Values given as mean ± standard errors (n = 3), statistical analyses by Dunnett T3 comparison test. * p<0.05; ** p<0.01; *** p<0.001.

Table 3 — ABTS$^{\bullet+}$radical scavenging activity of *Usnea* species

	TEAC* (mmol/L/Trolox)		
Samples	0.5 mg/mL	1 mg/mL	2 mg/mL
U. lapponica	1.17±0.17^{c}	1.51±0.21^{b}	2.38±0.24^{a}
U. intermedia	1.29±0.17^{b,c}	1.76±0.25^{d}	2.51±0.13^{a}
U. florida	0.91±0.11^{e}	1. 50±0,.21^{b}	2.24±0.27^{a}
*BHT***	2.50±0.1^{a}	2.55±0.8^{a}	2.5±0.9^{a}

Values given as mean ± standard errors (n = 3), statistical analyses by Tukey comparison test. The same lower case letters (a–e) are not significantly (p>0.05) different.

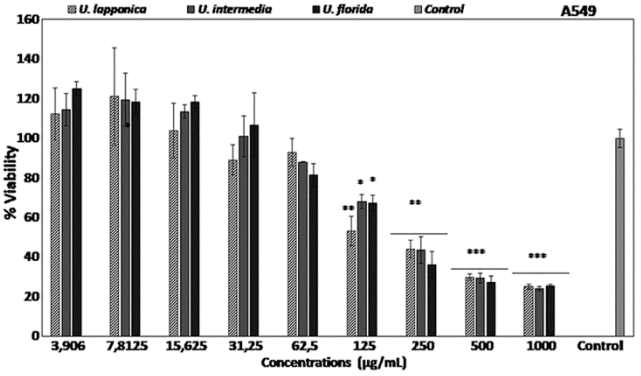

Fig. 2 — Cytotoxic activity results in A549 cell line

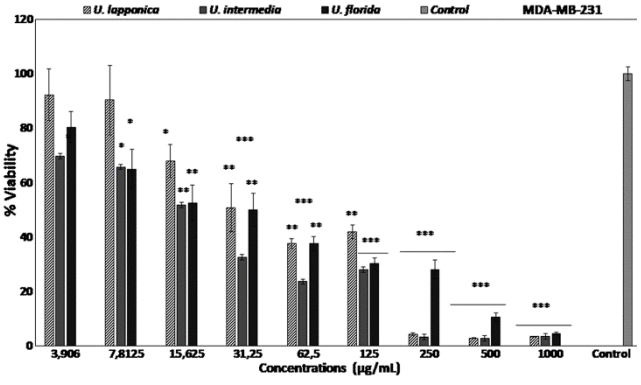

Fig. 3 — Cytotoxic activity results in MDA-MB-231cell line

on the A549 cancer cell line were 130.72±16.70 µg/mL, 178.00±33.26 µg/mL, and 204.19±28.18 µg/mL respectively (Table 4).

In MDA-MB-231 cells, the highest toxicity was determined in the *U. intermedia* extract with a vitality value of 32.70% at 31.25µg/mL, and the IC_{50} value of

Table 4 — IC50 values of extracts on A549 and MDA-MB-231

	IC50 (µg/mL)	
Extracts	A549	MDA-MB-231
U. lapponica	130.72±16.70	42.44±9.80
U. intermedia	204.19±28.18	16.43±4.64
U. florida	178.00±33.26	45.48±8.43

U. intermedia was 16.43±4.64 µg/mL (Table 4). Also, no similar toxic effects were observed in other species at the same concentration. At 15.625 µg/mL, lichen species could not reduce the viability below 50%. It was noted that *U. lapponica* (3.45%), *U. intermedia* (3.60%), and *U. florida* (4.57%) were highly cytotoxic to cells at a concentration of 1000 µg/mL (Fig. 3).

Antimicrobial activity assay

The *Usnea* methanol extracts antibacterial and antifungal activity was evaluated using the disc diffusion method against seven microorganisms, and the result is summarized in Table 5. The inhibitory zone diameters for the *U. lapponica*, *U. intermedia, and U. florida* were varied, between 7.0 and 13.0 mm for bacteria. Extracts from *U. lapponica, U. intermedia,* and *U. florida* showed similar antibacterial activity. *Usnea* extracts did not show antifungal effects on *Candida albicans*. The best antibacterial activity was obtained with *U. intermedia* extract against *M. luteus* (13.0 mm). *Usnea* extracts failed to exhibit antibacterial activity against *E. coli, P. mirabilis*, *E. aerogenes,* and *B. subtilis*. The activities of the extracts were not statistically significant with the standards (p>0.05). The outcomes indicated that commercial antibiotics (ampicillin/nystatin) had more potent activity than *Usnea* extracts, as shown in Table 5. No effect of the negative control DMSO on the tested microorganisms was observed (Fig. 4).

DNA cleavage assay

DNA cleavage assay of *Usnea* extracts was performed by agarose gel electrophoresis using plasmid DNA damage with irradiation. Figure 5 shows the electrophoretic pattern of plasmid DNA

Table 5 — Antimicrobial activity of methanol extracts of *Usnea* species (20 mg/mL) against tested microorganisms using disc diffusion methods

M. organism	Inhibition zone (mm)					
	U. lapponica	*U. intermedia*	*U. florida*	(-) Negative Control	(+) Positive Control	
					Ampicillin	Nystatin
E. coli	NI*	NI	NI	NI	17.0±0.12	
P. mirabilis	NI	NI	NI	NI	20.0±0.31	
M. luteus	11±0.21^{+}	13±0.33^{+}	11±0.17^{+}	NI	33.0±0.57^{++}	
E. aerogenes	NI	NI	NI	NI	11.0±0.17	
S. aureus	7±0.4^{a}	7±0.2^{a}	7±0.8^{a}	NI	20.0±0.24^{b}	
B. subtilis	NI	NI	NI	NI	12.5±0.11	
C. albicans	NI	NI	NI	NI		14.0±0.23

Values given as mean ± standard errors ($n = 3$), statistical analyses by Tukey comparison test. The same lower case letter (a–b) and the same symbol (+,++) are not significantly ($p>0.05$) different.

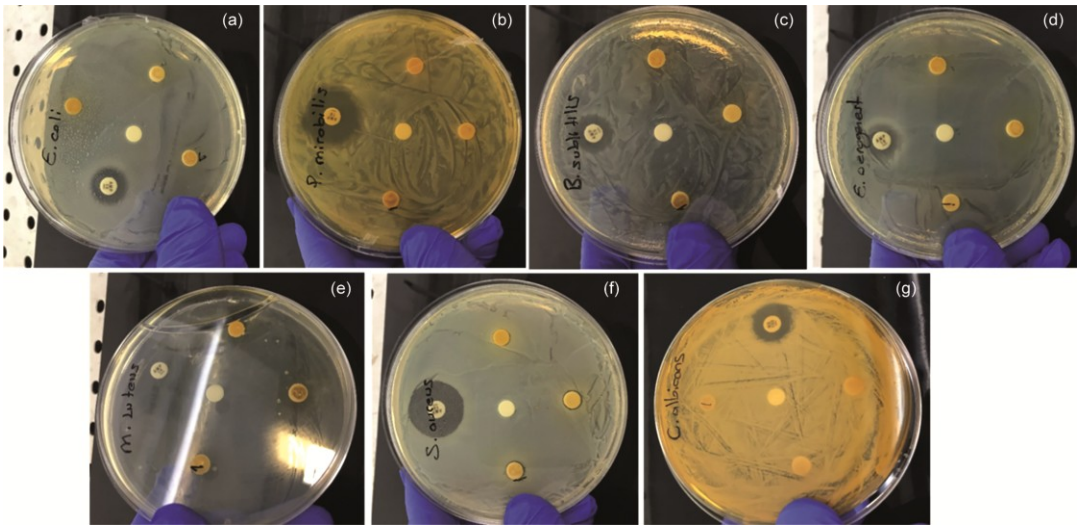

Fig. 4 — Antimicrobial activities of *Usnea* extracts (a. *Escherichia coli,* b. *P. mirabilis,* c. *B. subtilis,* d. *E. aerogenes,* e. *M. luteus,* f. *S. aureus,* g.*C. albicans*)

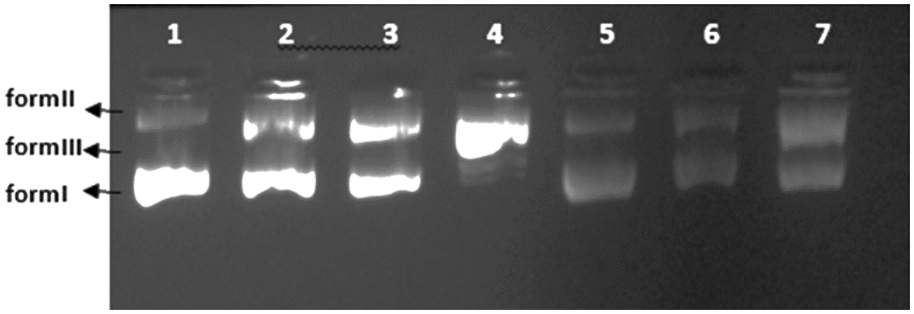

Fig. 5— line 1) Untreated PBR322 (control), line 2) pBR322 / UV, line 3) pBR322 / H_2O_2, line 4) pBR322+H_2O_2/ UV, line 5) pBR322 + *U. lapponica* extract + H_2O_2/ UV, line 6) pBR322 + *U. intermedia* extract + H_2O_2/ UV, line 7) pBR322 + *U. florida* extract + H_2O_2/ UV.

(PBR322) after UV-photolysis of H_2O_2. In gel electrophoresis, supercoiled circular DNA (form I) quickly migrates than open circular DNA (form II) and linear DNA (form III) forms. When the supercoiled form is broken, other form structures are generated. Untreated pBR322 (Control) showed two bands on gel electrophoresis (lane 1). The UV irradiation of pBR322 in the presence of H_2O_2 resulted in the cleavage of supercoiled circular DNA to open circular DNA and linear form (lane 4). The addition of *U. lapponica* (lane 5), *U. intermedia* (lane 6), and *U. florida* (lane 7) extracts to the reaction mixture of H_2O_2 suppressed the formation of linear DNA and induced a partial recovery of supercoiled circular DNA. The results showed that the methanol extract of *Usnea* had potent activity to protect DNA.

Discussion

In this study, three lichen species belonging to the genus *Usnea* were examined in terms of total phenol, total flavonoid content, amount of usnic acids with their antioxidant, antimicrobial, cytotoxic effects, and effects on DNA molecules.

The investigation revealed that *U. lapponica* exhibits the highest total phenol content, whereas *U. florida* demonstrates the highest total flavonoid amount (Table 1). In previous examinations of *Usnea* species, the total phenol amount of *U. florida*'s methanol and water extracts were reported as 10.04±0.0001 $mg_{GAE}/g_{extract}$ and 10.5±0.0004 $mg_{GAE}/g_{extract}$ respectively, in water and methanol extracts[29]. In this research, the total phenol content in the 80% methanol extract for *U. florida* was determined as 85.15±2.12 $mgGAE/g_{extract}$. Another investigation focusing on *U. intermedia* involved the preparation of ethanol, methanol, and acetone extracts, with the methanol extract revealing a total phenol content of 2.397±0.3 $mg_{GAE}/g_{extract}$ in the dry lichen sample[30]. Conversely, findings for the same species indicated a total phenol content of 73.74±1.62 $mg_{GAE}/g_{extract.}$ HPLC analyses were conducted to quantify usnic acid levels, revealing the highest content in *U. lapponica* at 3345.9±92.18 mg/g (Table 1, Fig. 1). Usnic acid amounts in other extracts were determined as 1337.57 mg/g in *U. intermedia* and 842.95 mg/g in *U. florida*. In a study by Cansaran *et al.,* (2006), *U. florida* was declared to contain usnic acid and vulpinic acid, with a usnic acid amount of 236±0.37 mg/g[31]; however, our investigation found the usnic acid content in *U. florida* to be 842.95±56.56 mg/g. Another study assessing usnic acid content in *U. intermedia* reported the amount in the methanol extract, prepared from a 1-gram lichen sample, as 0.94±0.04 mg/g_{plant}[30]. Out of all the examined samples, the *U. florida* extract showed the greatest level of activity. In assessing the antioxidant activity of *Usnea* species, our study utilized the $DPPH^{\bullet}$ radical scavenging method for the first time in lichen species. Oran *et al.,* (2016) investigated the radical scavenging effects of *U. intermedia*, *U. filipendula*, and *U. fulvoreagens* using the ABTS method[30]. Their findings indicated a superior effect in the *U. intermedia* methanol extract compared to extracts prepared with other solvents. In our study, the ABTS radical scavenging effect in the *U. intermedia* methanol extract at a concentration of 1 mg/mL was detected as 1.76±0.25 mmol/L/Trolox, contrasting with Oran's reported value of 141.6±6.0 mg TE /100 g_{plant}[30]. When comparing the antioxidant activity of our lichen sample with that of various plants, it is evident that lichens exhibit comparable antioxidant activity to plants[32].

In this investigation, *U. lapponica* emerged as the species with the highest usnic acid content, displaying elevated inhibition in the A549 cell line with the lowest IC_{50} value (130.72±16.70 µg/mL) compared to other extracts. Conversely, in the MDA-MB-231 cell line, *U. intermedia*, despite having lower usnic acid content than *U. lapponica*, exhibited potent inhibition with the lowest IC_{50} value (16.43±4.64 µg/mL) (Table 4). While numerous studies have explored the cytotoxic effects of *Usnea* genus species using various methods[33,34], our study marks the first to delineate the effects of the examined extracts on A549 and MDA-MB-231cell lines.

In various research investigations, *Usnea* species have exhibited antimicrobial activity against diverse microorganisms. In a study evaluating the antimicrobial effects of *U. florida*, bacteria such as *E. coli*, *S. aureus*, *E. fecalis*, *P. mirabilis*, *P. aeruginosa*, *B. subtilis*, and *B. megaterium* were employed. *U. florida* specifically demonstrated antimicrobial activity against *B. subtilis* and *B. megaterium*, with no discernible effects observed on other tested bacteria. Similarly, *U. longissima* exhibited antimicrobial activity exclusively against *B. subtilis* and *B. megaterium*, with no effects noted on other tested bacteria[31]. In our study, the *U. intermedia* extract exhibited the highest efficacy against the *M. luteus* bacterial strain. Moreover, none of the extracts showed any effect on *C. albicans* (Fig. 4).

Lichen extracts' interactions with plasmid DNA (PBR322) were evaluated using three forms: Form I (supercoiled), Form II (open ring), and Form III (linear). Exposure to UV rays and H_2O_2 can induce breaks in the supercoiled circular DNA (Form I), leading to changes in density and progression and the formation of Form II and Form III. Previous studies have reported DNA protective activity in various lichen species[28]. Treatment with UV, H_2O_2, and DNA resulted in alterations in the activity and density of Form II and Form I. Form I concentration decreased, and Form II density increased, accompanied by the appearance of Form III due to double-strand breaks. Lichen extracts interacted with PBR322, mitigating damage induced by UV and H_2O_2, similar to the control group. Consequently, the findings indicate

that lichen extracts have the ability to protect DNA from UV and H_2O_2 (Fig. 5).

Traditional uses of species belonging to the genus *Usnea* have been practiced for many years in the treatment of diseases by utilizing various medicinal properties of the genus. These lichens, which have been used for different health problems in every culture, have an important place among natural treatment methods. Especially *U. florida* and *U. intermedia*, which have traditional uses, as well as the activities of *U. lapponica* used in this study, suggest promising applications for their extracts or secondary metabolites in pharmaceutical, cosmetic, and industrial products.

Conclusion

In our research, *Usnea* species with traditional uses showed high toxicity against breast cancer cell lines. We aimed to associate the compounds responsible for the activities with more comprehensive studies. Notably, no discernible correlation was observed among the cytotoxic effect, antioxidant activity, and antimicrobial activity. While usnic acid has been implicated as the primary contributor to the observed activity, it is inferred that additional secondary metabolites inherent in the *Usnea* species also play a role. Despite the plethora of studies in the literature concerning various *Usnea* lichen species, there is a conspicuous dearth of investigations specifically concentrating on the biological activities of *U. lapponica*. This pioneering study, elucidating the cytotoxic potential of *U. lapponica* for the first time, contributes crucial data to the extant scientific discourse.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

MK and ZK formulated the experimental design for the present research. MK, GNİ, Sİ, ZK, GŞK, and AC executed the experiments. ZK, Sİ, and GŞK drafted and revised the manuscript. All authors have come to a consensus on approving this submission after a meticulous reading and approval process.

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