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Exploring The Therapeutic Potential of *Xanthoparmelia pulla* (Ach.) O. Blanco, A. Crespo, Elix, D. Hawksw. & Lumbsch (Parmeliaceae)

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Abstract

Aim of study: This study aims to investigate the antioxidant effect and potential toxicity of the methanol extract of *X. pulla* species in Turkey and to identify the secondary metabolites of this species.

Area of study: Samples were collected from Yozgat Bozok University East Campus.

Material and method: Detection of secondary metabolites in *X. pulla* was performed using HPLC. Antioxidant activity was evaluated using DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ methods. Cytotoxicity was evaluated using MTT assay in MCF-7 and MDA-MB-231 cell lines.

Main results: The highest secondary metabolite was salazinic acid (37.23±9.21 mg/extract). Total phenol content was 172.77±10.50 mg GAE/g and flavonoid content was 9.25±1.32 mg CA/g. The extract showed 87.41% DPPH$^{\bullet}$ radical scavenging activity at 2 mg/mL concentration and effectively neutralized ABTS$^{\bullet+}$ radical at all concentrations. In terms of cytotoxicity, MCF-7 cell viability was inhibited by 66.84% at 125 µg/mL and MDA-MB-231 cell viability was inhibited by 39.42% at 250 µg/mL.

Research highlights: This study reveals the biological activity of X. pulla and its potential for natural product based drug development.

Keywords: *Xanthoparmelia pulla*, HPLC, Salazinic acid, Antioxidant, Cytotoxicity

Xanthoparmelia pulla'nın (Ach.) O. Blanco, A. Crespo, Elix, D. Hawksw. & Lumbsch'un (Parmeliaceae) Terapötik Potansiyelinin Araştırılması

Öz

Çalışmanın amacı: Bu çalışma, Türkiye'de bulunan *X. pulla* türünün metanol ekstresinin antioksidan etkisi ve potansiyel toksisitesini araştırmayı ve bu türün sekonder metabolitlerini tanımlamayı amaçlamaktadır.

Çalışma alanı: Örnekler Yozgat Bozok Üniversitesi Doğu Kampüsü'nden toplanmıştır.

Materyal ve yöntem: X. pulla'daki sekonder metabolitlerin tespiti HPLC kullanılarak gerçekleştirilmiştir. Antioksidan aktivite, DPPH$^{\bullet}$ ve ABTS$^{\bullet+}$ yöntemleri kullanılarak değerlendirilmiştir. Sitotoksisite, MCF-7 ve MDA-MB-231 hücre hatlarında MTT deneyi kullanılarak değerlendirildi.

Temel sonuçlar: En yüksek sekonder metabolit salazinik asit (37.23±9.21 mg/ekstre) olarak tespit edilmiştir. Toplam fenol içeriği 172.77±10.50 mg GAE/g, flavonoid içeriği ise 9.25±1.32 mg CA/g'dır. Ekstre, 2 mg/mL konsantrasyonda %87.41 DPPH$^{\bullet}$ radikal süpürme aktivitesi göstermiş, ABTS$^{\bullet+}$ radikalini ise tüm konsantrasyonlarda etkili şekilde nötralize etmiştir. Sitotoksisite açısından, 125 µg/mL'de MCF-7 hücre canlılığı %66.84, 250 µg/mL'de MDA-MB-231 hücre canlılığı %39.42 oranında inhibe edilmiştir.

Araştırma vurguları: Bu çalışma, *X. pulla*'nın biyolojik aktivitesi ve doğal ürün bazlı ilaç geliştirme potansiyelini ortaya koymaktadır.

Anahtar Kelimeler: *Xanthoparmelia pulla*, HPLC, Salazinic acid, Antioksidan, Sitotoksisite

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Introduction

Lichens are symbiotic forms of fungi that can thrive in a broad spectrum of natural ecosystems, ranging from limestone rocks to siliceous soils, and from aquatic environments to tree trunk and branch barks, as well as leaves. They are established through the symbiotic relationship between fungi and algae, displaying distinct morphological, anatomical, and physiological characteristics (Duman, 2008; Karamanoğlu, 1971). A notable attribute of the symbiotic life of these organisms is their capability to produce diverse "lichen substances" (Lawrey, 1986; Yamamoto et al., 2015).

While lichens synthesize these metabolic products in a unique way, fungi and algae alone do not possess this ability (Tanker et al., 2007). In response to any stressful situation, lichens can produce both secondary and primary metabolites through specialized metabolic pathways (Valarmathi et al., 2009). The majority of secondary metabolites are generated by fungi and constitute a significant portion of the organic compounds found in lichens. Although these metabolites are typically stored on the surface of hyphae, they are not located inside the cell (Ranković and Kosanić, 2019). Research on lichen-derived secondary metabolites has revealed their broad spectrum of biological activities. These compounds exhibit significant antimicrobial effects, including antibacterial, antiviral, and antifungal properties, as well as notable antioxidant, antiproliferative, and anti-inflammatory potentials. Their diverse bioactive functions highlight their potential applications in pharmaceutical and biomedical fields (Jeon et al., 2009). Observations have revealed the cytotoxic properties of lichens in many cancer cell lines, and it has been established that the cytotoxic potency of lichens is greater in cancer cells compared to healthy cells (Türk et al., 2003; Solárová et al., 2020).

The genus *Xanthoparmelia* (Vain.) Hale (Parmeliaceae) encompasses more than 800 lichen species distributed globally. A predominant characteristic of species within this genus is the presence of a yellow-green upper surface, often containing compounds such as usnic acid and isousnic acid in their structural composition. In European species, five distinct chemical structures have been identified: constipatic acid, fumarprotocetraric acid, norstictic acid, salazinic acid, and stictic acid (Melo et al., 2011; Lücking et al., 2016; Pérez-Vargas et al., 2024). Traditionally, lichens from the *Xanthoparmelia* genus have exhibited effectiveness in addressing conditions such as arthritis, rheumatism, chronic pain, swelling, and heightened menstrual bleeding (Rankovic, 2019). Notably, *Xanthoparmelia pulla* (Ach.) O. Blanco, A. Crespo, Elix, D. Hawksw. & Lumbsch species within this genus have thus far been investigated solely for their antifungal activity (Aslan et al., 2006). Lichens have a longstanding history of traditional medicinal use across various countries and are well known for their therapeutic properties (Shibata et al., 1948). This species is broadly distributed in Turkey and is locally referred to as Esmergüzeli (John and Türk, 2017).

This research aims to determine the total phenolic and flavonoid amounts in the methanol extract of *X. pulla*. The analysis involves the quantification of bioactive compounds using spectrophotometric methods to provide insight into the antioxidant potential and phytochemical composition of the extract. Additionally, the study aims to identify and quantify commonly encountered lichen acids using High-Pressure Liquid Chromatography (HPLC). Furthermore, the antioxidant and cytotoxic effects of *X. pulla* were assessed through in vitro experiments, contributing to a broader understanding of its potential pharmacological applications and bioactive properties.

Material and Methods

Determination of Samples

The specimen used in the study was collected from Yozgat Bozok University, East Campus, 39°46'21" N., 34°48'14" E. coordinates, 1353 m. altitude on 25.11.2022. Identification keys from flora books and articles, such as "Flechten Flora" (Wirth et al., 1995) and "The Lichen Flora of Great Britain and Ireland" (Purvis et al., 2002), were utilized to identify lichen samples collected during the field study. External

morphological features were determined using the Olympus SZX16 Stereo Microscope, while anatomical features were examined using the Olympus BX53 Light Microscope. Additionally, digital photographs of the samples were captured using the Olympus DP25 digital camera integrated with the Olympus SZX16 and Olympus BX53 (Figure 1). During the species identification process, crucial characteristics such as spore sizes, ascus sizes, hymenium heights, and paraphysis widths were meticulously measured with an ocular micrometer. These precise measurements greatly contributed to the species identification. Moreover, more detailed analyses were conducted using chemical reagents in species identification studies. After the collected samples were transformed into herbarium material, they were carefully placed in envelopes, recorded, and stored in the Yozgat Bozok University Faculty of Arts and Sciences Lichen Herbarium [Herbarium number: MK 137]. This storage method ensures the availability of samples for future research.

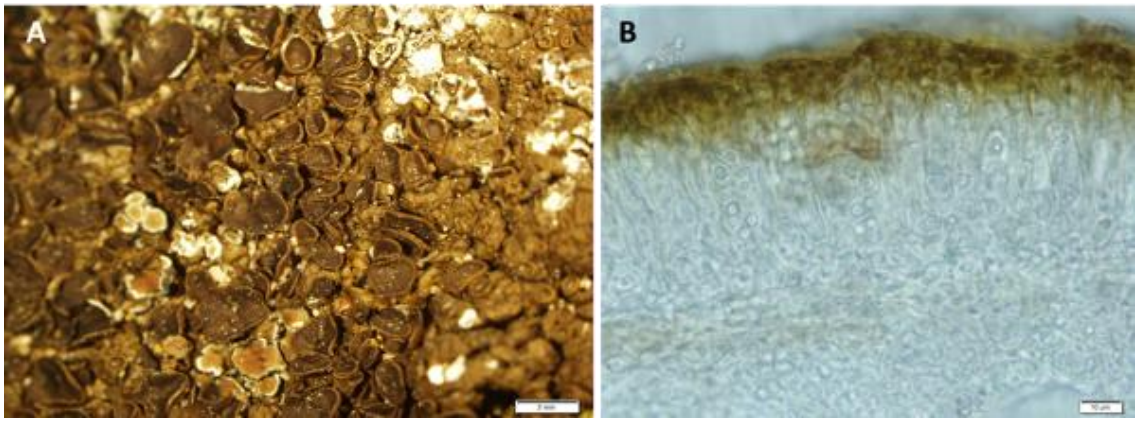

Figure 1. Thallus of *Xanthoparmelia pulla* (A) and its microscopic image (B)

Preparation of Extracts

Three extractions were performed on a 10-gram sample of *Xanthoparmelia pulla* using 80% methanol in a shaking water bath at a temperature 37 ºC for 8 hours. The obtained extracts were pooled and subjected to concentration under reduced pressure utilizing a rotary evaporator set at a temperature of 37–38 ºC. Following this process, the extracts underwent freeze-drying and desiccation to ensure the removal of residual moisture. Finally, they were preserved at -20 ºC under controlled conditions until further analytical procedures were conducted.

Quantification of Total Phenolic Compounds

Total phenolic content of the extracts was determined by the Folin-Ciocalteu method, a widely used and accepted spectrophotometric method for the quantitative analysis of polyphenolic compounds. The amount of phenolic compounds was calculated based on the gallic acid calibration curve and the results were standardized and presented as gallic acid equivalents (GAE) to facilitate comparative analysis. This analysis was performed in accordance with the standardized procedure established by Singleton et al. (1999). A 100 µL portion of the sample solution was mixed with 500 µL of Folin-Ciocalteu reagent in a 10 mL vessel containing 6 mL of distilled water. After one minute, a volume of 1.5 mL of a 20% solution of Na_2CO_3 in water was introduced, and subsequently, 10 mL of water was added to achieve a homogeneous mixture. A control was employed, consisting of a reagent mixture devoid of extract. Following a 2-hour incubation at 25ºC, the absorbance of the reaction mixture was recorded at 760 nm using a spectrophotometer to determine its optical density. The quantification of total phenolic content was carried out by measuring the absorbance of the samples and correlating the values with a gallic acid calibration curve. The results were then expressed in terms of gallic acid equivalents

(GAE) to standardize phenolic concentration across samples. To enhance the reliability and reproducibility of the findings, all experiments were conducted in triplicate under standardized conditions, ensuring consistency across measurements and minimizing experimental variability. The obtained data were statistically analyzed and presented as mean values along with their respective standard deviations to reflect the precision and reproducibility of the measurements.

Quantification of Total Flavonoid Compounds

The examination of flavonoids in the extracts was conducted using an adapted technique established by Singleton et al. (1999). These modifications facilitated precise quantification of flavonoid content and were employed to assess the flavonoid composition of the present extracts. To achieve this objective, at the beginning (t=0), 1 mL of the extracted sample was mixed with 0.3 mL of a solution containing 5% sodium nitrite ($NaNO_2$). At time t=5, a volume of 0.3 mL of a solution containing 10% aluminum chloride ($AlCl_3.6H_2O$) was introduced. At t=6, a 2 mL portion of a 1 M sodium hydroxide (NaOH) solution was added and thoroughly mixed. Ultimately, a total of 2.4 milliliters of water was introduced. The procedures employed were successful in determining the flavonoid content in the sample. Absorbance measurements were conducted at a wavelength of 510 nm. The total flavonoid content in the extracts was measured in milligrams of catechin acid (CA) per gram of extract, which is equivalent to catechin. The calibration curve for catechin was created similarly, utilizing ethanol. The measurements were replicated in triplicate, and the resultant values were computed as the arithmetic mean.

Secondary Metabolite Determination By HPLC

Samples of lichen extracts were prepared using a 70% methanol solution. The analyses were conducted using the Shimadzu LC-20AT HPLC system. The PDA spectrophotometric detector was employed with a solvent mixture of methanol-water-acetic acid (85:15:5, v/v/v) for the analyses. The flow rate was set at 1 mL/min, and the standards were dissolved in acetone. The column used in HPLC analyses was Thermo Scientific brand, with a particle size of 5 microns and 250x4.6 mm. Analyses were performed at an operating temperature of 30°C. The standards used in the HPLC analysis were: usnic acid, gyrophoric acid, psoromic acid, fumarprotocetraric acid, protocetraric acid, stictic acid, norstictic acid and salazinic acid. These standards were prepared at concentrations of 25 ppm, 50 ppm and 100 ppm. The chromatographic profiles of the standards prepared at 100 ppm concentration are given in Figure 2.

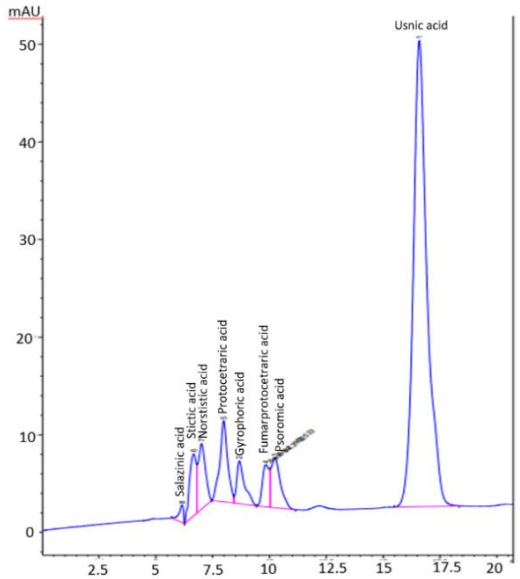

Figure 2. Chromatogram of standards

Assessment of DPPH• Radical Scavenging Activity

The capacity of the extracts to eliminate DPPH• radical was analyzed by examining the studies in the existing literature and applying appropriate methods (Gyamfi et al., 1999; Efe et al., 2021; Karaçelik et al., 2022; Karaçelik et al., 2022a). Before the study, a solution was prepared by mixing 50 millimolar (mM) Tris-HCl buffer with a pH of 7.4 and 1 milliliter (mL) of 0.1 mM methanol. Later on, this solution was merged with DPPH•. A control was used consisting of a reagent mixture without any extract. After being incubated for 30 minutes in a controlled setting with limited light exposure

at room temperature, the samples were analysed for their absorbance values at a wavelength of 517 nm. The inhibition percentage was determined by applying the formula outlined in Equation 1.

$$\%\ inhibition = \left[\frac{(Abs\ Control - Abs\ Sample)}{Abs\ Control}\right] x100 \quad (1)$$

To enhance the precision and dependability of the findings, each analysis was conducted in triplicate under uniform experimental conditions. The obtained data were then averaged to generate a representative value, minimizing potential variability and ensuring consistency in the results.

Evaluation of $ABTS^{\bullet+}$ Radical Scavenging Activity

The assessment of $ABTS^{\bullet+}$ radical scavenging efficacy in the extracts adhered to the experimental protocol elucidated by Re et al. (1999). The $ABTS^{\bullet+}$ radical aqueous solution was prepared by dissolving 36 mg of $ABTS^{\bullet+}$ and 6.6 mg of $K_2S_2O_8$ in 10 µL of water, and then allowing it to sit in darkness for 12-16 hours. The absorbance of the substance was calibrated to 0.700 (±0.030) at a wavelength of 734 nm while maintaining a room temperature environment. The produced radical solution (900 µL) was combined with the extract solutions (100 µL), and reaction kinetics measurements were performed at 734 nm for 30 minutes, with measurements taken every minute. The inhibitory percentages, determined according to concentration, were computed as Trolox equivalent antioxidant capacity (TEAC). The experiments were replicated thrice in a manner that was not influenced by any external factors, and the mean value of the outcomes was computed.

Evaluation of Cytotoxic Effects of Extracts Using the MTT (3-(4,5-dimethylthiazol–2-yl) diphenyltetrazoliumbromide) Assay

The cytotoxic effects of 80% methanol extract on MCF-7 and MDA-MB-231 cell lines were analyzed by MTT colorimetric assay to evaluate cell viability. This method helped to determine the effects of the extract on cell growth and life cycle by measuring the activity of cell metabolism. In this study, the cells were cultured under optimal conditions in an appropriate growth medium, ensuring a controlled environment for accurate evaluation of cell viability and response to the extract. The cells in the flask were enumerated and then moved to a 96-well microplate, with each well containing a seeding volume of 100 µL and 10,000 cells. After 24 hours, the medium that was covering the cells attached to the plate was removed. Following that, 100 µL of extracts produced at various concentrations ranging from 3.9 to 1000 µg mL^{-1} were introduced onto the plate. The plates were placed in a carbon dioxide incubator set at a temperature of 37 °C for 48 hours. A 0.5 mg mL^{-1} MTT solution was made by diluting the stock MTT solution in sterile PBS. This solution was then applied to the 96-well plates. Following a 3-hour period of incubation in the incubator, the media of the cell plate was removed and replaced with 100 µL of DMSO (Dimethyl sulfoxide), which was then thoroughly mixed. The optical densities of the cells in the plate were subsequently assessed at a wavelength of 540 nm using Bio-Rad brand ELISA equipment manufactured in the United States. The absorbance values of the control wells were also recorded, and the average values were computed. These values were considered as the baseline for 100% living cells. The absorbance values obtained from wells treated with the solvent and extract were analyzed to determine cell viability, expressed as a percentage relative to the control group, following the formula in Equation 2 (Şeker Karatoprak et al., 2019).

$$\%\ Cell\ viability = \left(\frac{Concentration\ O.D.}{Control\ O.D.}\right) x100 \quad (2)$$

Statistical Analysis

Variance homogeneity was assessed using Levene's test, ANOVA was used to compare more than two groups, and Dunnett's T3 test was used for multiple comparisons. Analyses were performed using SPSS (Version 11.0), and the significance level was determined as $p < 0.05$.

Results and Discussion

The phenolic content, determined spectrophotometrically, was quantified in terms of gallic acid equivalents. The collected findings are displayed in Table 1.

Table 1. Total phenol and flavonoid amount of *X. pulla* extract

Extract	Yield %	Total Phenol [$mg_{GAE}/g_{extract}$]	Total Flavonoid [$mg_{CA}/g_{extract}$]
X.pulla	7.21	172.77±10.50	9.25±1.32

Results are given as Mean ± SD; n=3

The *X. pulla* extract exhibited a total phenolic content of 172.77±10.50 mg GAE/extract. The extract's total flavonoid content was determined by converting it into an equivalent amount of catechin. These analyses are a commonly employed technique for quantifying the amount of flavonoids in extracts and evaluating their possible biological impacts. The *X. pulla* extract was found to have a total flavonoid concentration of 9.25±1.32 mg CA/gextract, as indicated in Table 1. The phenolic and flavonoid contents of the *X. stenophylla* were examined by analyzing the acetone and chloroform extracts. The phenolic content of acetone extracts was quantified as 32.15±0.85 µg mL^{-1} GAE, whereas the total phenolic content of chloroform extract was measured to be 42.58±0.47 µg mL^{-1} GAE. The acetone and chloroform extracts had total flavonoid levels of 113.56±1.02 and 24.53±1.15 µg mL^{-1} GAE catechin equivalent, respectively. It was shown that the *X. pulla* had a larger total phenol content compared to the *X. stenophylla* (Ach.) Ahti & D. Hawksw. species, while the flavonoid content was lower. The phenol and flavonoid contents of *X. conspersa* (Ehrh. ex Ach.) Hale were analysed by examining the ethanol and acetone extracts simultaneously. The phenolic content of the ethanol extract was determined to be 67.72±0.01 µg mL^{-1} GAE, whereas the total phenolic content of the acetone extract was found to be 209.92±0.01 µg mL^{-1} GAE. The ethanol extract had a flavonoid content of 68.68±0.03 µg mL^{-1} GAE, but the acetone extract had a total flavonoid concentration of 83.60±0.02 µg mL^{-1} GAE (Karaahmet et al., 2019). Upon comparing the results, it was found that the *X. pulla* taxa exhibited a higher concentration of total phenols, whereas the amount of flavonoids was lower. Studies on several species within the same genus have revealed that the chemical makeup of these species might differ, leading to distinct biological activity.

In the study, high pressure liquid chromatography (HPLC) was used to determine the amounts of lichen acids (Table 2). With this method, a sensitive and reliable quantitative evaluation of various acids from lichen extracts was achieved.

Table 2. Quantification of Lichen Acids in *X. pulla* Extract (mg/L, ppm)

Lichen acid	Concentration in *X. pulla*
Usnic acid (1)	2.42
Gyrophoric acid (2)	9.42
Psoromic acid (3)	4.29
Fumarproto cetraric acid (4)	-
Protocetraric acid (5)	2.49
Stictic acid (6)	-
Norstictic acid (7)	3.58
Salazinic acid (8)	37.23

Figure 3 presents the chromatogram obtained from the HPLC analysis of *X. pulla*, conducted to evaluate the acid content of the extract. *Parmelia pulla* Ach., a synonym for *X. pulla*, is known to contain various compounds, including divaricatic acid, gyrophoric acid, jiroforic acid, glomelliferic acid, glomellic acid, loxodellic acid, 4-O-methylolivetoric acid, microphyllinic acid, oxostenosporic acid, perlatolic acid, and stenosporic acid (Culberson et al., 1977). However, the chemical composition of these compounds can vary significantly depending on the geographic origin of the lichen samples and the extraction methods used.

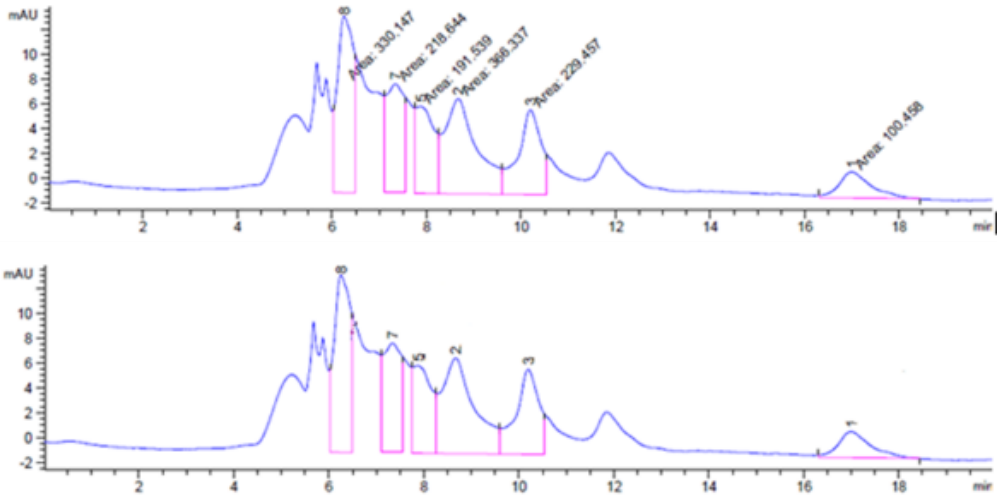

Figure 3. HPLC analysis chromatogram of *X. pulla*

The extracts were examined for their ability to scavenge the DPPH• radical within the concentration range of 0.00195-2 mg mL^{-1}. The extract shown concentration-dependent radical scavenging action at healthy pH levels. The *X. pulla* extract demonstrated a radical scavenging ability of 87.41% at the greatest concentration. However, this percentage decreased to 24.24% when the concentration was reduced to 0.125 mg mL^{-1} (Figure 4). According to reports, the methanol extract of *Neofuscelia pulla* (Ach.) Essl. does not exhibit any DPPH• radical scavenging action (Aslan et al., 2006).

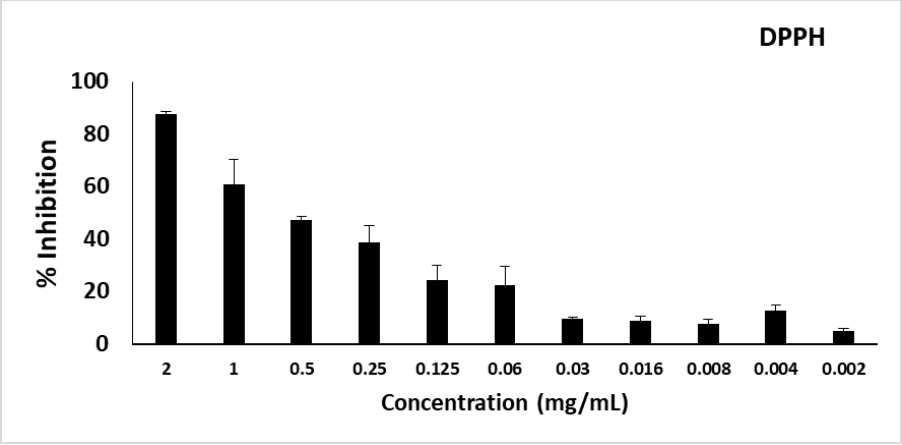

Figure 4. DPPH• radical scavenging activity of *X. pulla* extract
Values given as mean ± sd are stated within ± 95% confidence interval (n=3)

The study evaluating $ABTS^{+\bullet}$ radical scavenging activity is shown in Figure 5. While there is no existing work specifically evaluating the $ABTS^{+\bullet}$ radical scavenging activity of *X. pulla*, the antioxidant capacity of ethanol and acetone extracts from the *X. conspersa* was examined using the $ABTS^{\bullet+}$ radical scavenging activity technique. Although the acetone extract did not show any noticeable antioxidant effect, it was determined that the ethanol extract had a radical scavenging capacity of 209.00±5.55 µg GAE mL^{-1} (Karaahmet et al., 2019).

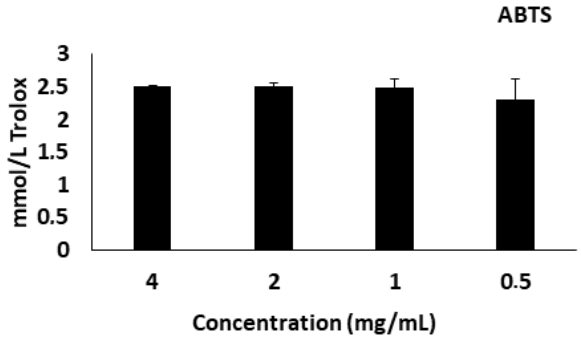

Figure 5. ABTS$^{\bullet+}$ radical scavenging activity of *X. pulla* extract
Values given as mean ± standard are stated within ± 95% confidence interval (n=3).

The extract of *X. pulla* showed a significant inhibitory impact on the viability of the MCF-7 cell line at doses of 125, 250, 500, and 1000 µg mL^{-1} ($p<0.001$). The viability was found to be 33.16% at a concentration of 125 µg mL^{-1}. Significance was observed at a p-value of less than 0.05 in the concentration range of 7.81-62.5 µg mL^{-1}, when compared to the control. No notable disparity in viability was detected when comparing the control to a concentration of 3.9 µg mL^{-1}. Figure 6 displays the toxicity outcomes on the MCF-7 cell line.

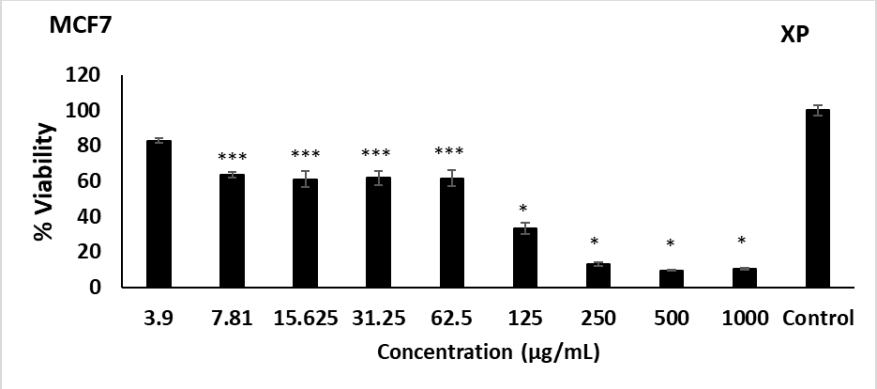

Figure 6. Toxicity of *X. pulla* extract on MCF-7 cell line.
Values given as mean ± SD are stated within ± 95% confidence interval (n = 3). Statistically significant differences *p<0.001; **p<0.01; *** indicated as p<0.05

The *X. pulla* extract exhibited a substantial reduction in cell viability within the MDA-MB-231 cell line, notably at concentrations of 500 and 1000 µg mL^{-1} ($p<0.001$). At a concentration of 250 µg mL^{-1}, the viability was determined to be 60.58%, and statistical analysis indicated a significant difference ($p<0.05$) compared to the control. However, within the concentration range of 3.9-62.5 µg mL^{-1}, the viability did not manifest a statistically significant disparity when compared to the control. Figure 7 presents the observed toxicity effects on the MDA-MB-231 cell line.

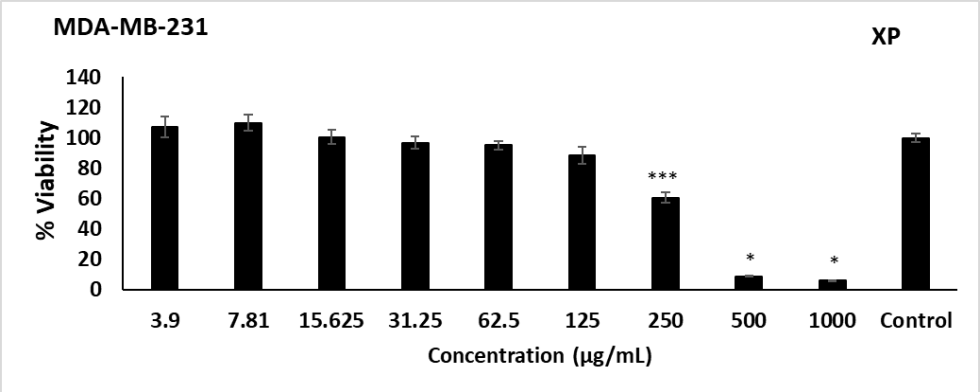

Figure 7. Toxicity of *X. pulla* extract on MDA-MB-231 cell line

Values given as mean ± SD are stated within ± 95% confidence interval (n=3). Statistically significant differences *p<0.001; **p<0.01; *** indicated as p<0.05.

Scabrosine esters obtained from *X. scabrosa (*Taylor) Hale have been found to exhibit strong cytotoxic effects against the MCF-7 cell line, a human breast cancer cell line, in MTT experiments. The IC50 value, which represents the concentration at which these drugs demonstrate action, was found to be around 1 µM (Ernst-Russel et al., 1999). In the investigation conducted by Öztürk et al. (2021), the lyophilized extracts derived from *X. somloensis* (Gyeln.) Hale were found to possess an IC50 value of 58.8 ± 0.23 µg mL^{-1} in the MCF-7 cell line, while in the MDA-MB-231 cell line, the IC50 value was determined to be 45.8 ± 10.68 µg mL^{-1}. Notably, the study highlighted that the cytotoxic activity of the *X. pulla* extract was more pronounced in the MCF-7 cell line, underscoring its potent impact in this specific context.

Conclusions

While extensive research exists in the literature on various *Xanthoparmelia* lichen species, investigations into the biological activities of *X. pulla* are notably scarce. This study aimed to address this gap by not only quantifying the total phenol and total flavonoid content of secondary metabolites from *X. pulla* but also exploring their antioxidant properties and cytotoxic effects on two distinct breast cancer cell lines. This research represents the first comprehensive examination of the biological properties of the *X. pulla* species, revealing its pronounced cytotoxic activity specifically against the MCF-7 cell line. In light of these findings, it is imperative to advance further studies to identify and assess the specific compounds responsible for this observed effect. Such initiatives are crucial for a more in-depth understanding of the therapeutic potential inherent in lichens traditionally used in medicinal practices.

Ethics Committee Approval

N/A

Peer-review

Apparently reviewed

Author Contributions

Conceptualization: S.K., M.K., and S.İ.; Investigation: S.K., M.K., S.İ., and Z.K.; Material and Methodology: S.K., M.K., S.İ., G.Ş.K., Z.K., and M.Ç.; Supervision: S.K., M.K., S.İ., and Z.K.; Visualization: Z.K., S.İ., G.Ş.K., and M.Ç.; Writing-original Draft: S.K., M.K., G.Ş.K., and Z.K.; Writing-review & Editing: S.K., M.K., G.Ş.K., and Z.K. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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