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INVESTIGATION OF ANTHRAQUINONE CONTENTS, DNA CLEAVAGE, DNA BINDING, CYTOTOXIC AND ANTIOXIDANT ACTIVITIES OF *XANTHORIA PARIETINA* SAMPLES

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ABSTRACT. In this study, *Xanthoria parietina* samples were collected from different regions of Türkiye like Yozgat (Xp3), Izmit (Xp14), and Kütahya (Xp20). Anthracenedione, anthraquinone (parietin) contents of the lichens were determined quantitatively by GC-MS and spectrophotometric methods. The interaction of lichen extracts with pBR322 DNA and CT-DNA was examined by performing an agarose gel electrophoresis method. The cell proliferative activities of *Xanthoria parietina* samples were tested against the colon cancer cell line (DLD-1) by MTT assay. As a results of the GC-MS and spectrophotometric analysis, the highest and the lowest parietin contents were found for Xp20 and Xp14 extracts, respectively. These results were supported by those of the DNA cleavage, binding, and toxicity studies. The Xp14 sample can be considered as a drug that could be a new approach to cancer treatment, as it has the lowest polyaromatic hydrocarbon content and is not toxic for the cell.

Keywords: *Xanthoria parietina; parietin; DNA cleavage; cytotoxicity; colon cancer, GC/MS*

INTRODUCTION

New pharmacologically effective synthetic drugs are usually accompanied by the emergence of new side effects. Hence, the discovery and use of phytochemicals as the savior of this situation has become widespread. Also, new lichens including a richer and different content from plants have been discovered as drug raw materials and DNA binding agents.

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Herbal medicines have been used for a long time in the treatment of diseases such as cancer [1-6]. They are vital owing to the production of unique substances including more than 800 aliphatic, cycloaliphatic, aromatic, polyaromatic and terpenic compounds [7]. Some studies report that these compounds possess antibiotic, antimicrobial, antiviral, anti-inflammatory, analgesic, and antipyretic qualities. A number of unique chemical agents of lichens have also been proven to be effective against various cancer models. In addition, lichens have been widely used as food, feed, perfume, spice, dye, and traditional medicine all over the world. Besides, many types of lichens have been utilized for the treatment of diseases and cancer in recent years There are more studies on the anticancer activity of secondary metabolites found in lichens. The most common seconder metabollite found in lichen species is usnic acid. There are at least 40 articles about it. Especially the Evernia prunastri species contains a lot of it. In the study conducted by [8], it was found that Atranorin secondary metabolite showed strong cytotoxic activity against brain cancer, breast cancer, cervical cancer, colorectal cancer, lung cancer, ovarian cancer, prostate cancer, melanoma cancer types (IC_{50} between 12.5 and 26.5 µg/ml) (except leukemia cell lines (IC_{50} = 93.5 µg/ml). In the study conducted by [9] the cytotoxic effects of metabolites isolated from the lichen species Flavocetraria cucullata, such as usnic acid, salazinic acid, squamatic acid, baeomysesic acid, d-protolichesterinic acid and lichesterinic acid, on several human cancer cells were evaluated by the MTT method. The cells in which the determined IC_{50} value for usnic acid was obtained activated the specific apoptotic signaling pathway and an increase in the apoptotic cell population was observed. In the study conducted by Singh et al.; the anticancer effect of usnic acid in human lung carcinoma A549 cells and possible molecular changes were evaluated. Usnic acid secondary metabolite significantly suppressed the proliferative effect of A549 cell line. Cell growth inhibition was associated with cell cycle arrest in G0 and G1 phase. Usnic acid decreased the expression of cyclin-dependent kinase (CDK)4, CDK6 and cyclin D1 and increased the expression of CDK inhibitor (CDKI) p21/cip1 protein. Thus, usnic acid caused an increase in apoptotic cells more than two-fold. The apoptotic effect of usnic acid was realized by increased poly(ADP-ribose) polymerase cleavage [10-15].

Xanthoria parietina is a leafy lichen in the Teloschistaceae family. Xanthoria species have been traditionally used for various purposes, including medicinal utilities (antipyretic and jaundice) in Anatolia. Due to the anthraquinone dyestuff, which is abundant in its structures [16-20], they can be used for colouring purposes. Parietin-containing anthraquinones are well-known as one of the common bioactive compounds of lichens. Numerous scientific studies have presented the chemical composition, enzyme inhibition activities of Xanthoria lichens cultivated worldwide, and biopharmacological properties

of their parent compound "parietin" [21-23]. However, only a few studies have investigated the biologically active species and potential biological activities of Xanthoria lichens grown in Turkey [24].

Parietin is an anthraquinone pigment usually isolated from some plants such as *Rheum ribes* and *Xanthoria parietina* (lichen species). This secondary metabolite is localized as a small extracellular crystal in the uppermost level of the upper cortex of lichens and plays a protective role owing to its strong orange-brownish coloration against sunlight [25-27]. Parietin was considered responsible for the antiproliferative, antibacterial, antifungal, antioxidant [28,29], In this literature study, it was shown that parietin showed cytotoxic effect but not genotoxic effect at low concentration in HepG2 cells. As a result, it is predicted that parietin may be a useful agent in combination with other drugs in the treatment of hepatocellular carcinoma and should be supported by more detailed studies [30]. In the study conducted by Dodurga et al., the effects of parietin on cytotoxicity, gene expression, migration, invasion and colony formation in neuroblastoma cells treated with parietin were investigated. As a result, it was stated that parietin could be used as an alternative, complementary and supportive agent together with other drugs in the treatment of neuroblastoma [31,32]. There are many studies showing that parietin is an anthraquinone that has promising effects in preventing the proliferation of cancer cells and tumor growth [33-35].

In this study, the chemical profile of *Xanthoria parietina* (Xp) belonging to the family of Teloschistaceae was analyzed. The examined *Xanthoria parietina* was collected from three cities that are placed in different regions of Türkiye like Yozgat (Xp3), Izmit (Xp14), and Kütahya (Xp20). Since the DNA cleavage and DNA binding properties of the Xp lichen species, which were collected from different regions of Turkey, have not been reported in the literature, this original study can fill this empty in the literature. Total phenolic and total flavonoid content of Xp samples assessed spectroscopically. Besides, the in vitro antiproliferative effects of the Xp compounds were tested using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

RESULTS AND DISCUSSION

GC-MS Analyses

The GC-MS results are listed in Tables (1-3). As a result, parietin, which has a flavonoid structure, was obtained 65.23% for Xp3, 58. 03% for Xp14, and 71.75% for Xp20. The related spectra are showed in Figure 1 Parietin (anthraquinone derivative) was the main component determined by GC-MS in three Xp extracts.

Table 1. GC-MS results of sample Xp3

No	RT (min)	Formula	Area%	Molecular	Name
1	5.444	$C_6H_{12}O_2$	4.35	116.158	4-hydroxy-4-methylpentan-2-one
2	46.597	$C_{18}H_{22}$	0.59	238.367	(2,3-dimethyl-3-phenylbutan-2-yl)benzene
3	49.035	$C_{18}H_{20}$	0.97	236.351	[(*E*)-2-methyl-4-phenylpent-3-en-2-yl]benzene
4	81.264	$C_{24}H_{38}O_4$	25.09	390.556	bis(2-ethylhexyl) benzene-1,2-dicarboxylate
5	86.202	C_{16} H_{12} O_5	65.23	284.268	1,8-Dihydroxy-3-methoxy-6-methyl-9,10-
6	90.575	$C_{20}H_{40}O$	2.33	296.540	(*E*)-3,7,11,15-tetramethylhexadec-2-en-1-ol
7	91.994	$C_{19}H_{33}Br$	1.44	373.400	3-bromoprop-2-ynyl hexadecanoate

Table 1 shows that the Xp3 sample contained 1,8-Dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone at the highest value of 65.23%. This is consistent with the spectrum result.

Table 2. GC-MS results of sample Xp14

No	RT (min)	Formula	Area%	Molecular	Name
1	5.501	$C_6H_{12}O_2$	4.05	116.158	4-hydroxy-4-methylpentan-2-one
2	45.591	$C_{18}H_{22}$	0.45	238.367	(2,3-dimethyl-3-phenylbutan-2-yl)benzene
3	47.035	$C_{18}H_{20}$	0.82	236.351	[(*E*)-2-methyl-4-phenylpent-3-en-2-yl]benzene
4	80.244	$C_{24}H_{38}O_4$	24.03	390.556	bis(2-ethylhexyl) benzene-1,2-dicarboxylate
5	85.212	C_{16} H_{12} O_5	58.03	284.268	1,8-Dihydroxy-3-methoxy-6-methyl-9,10-
6	89.504	$C_{20}H_{40}O$	1.23	296.540	(*E*)-3,7,11,15-tetramethylhexadec-2-en-1-ol
7	90.871	$C_{19}H_{33}Br$	0.94	373.400	3-bromoprop-2-ynyl hexadecanoate

Table 2 shows that the Xp14 sample contained 1,8-Dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone at the highest value of 58.03%. This is consistent with the spectrum result**.**

Table 3. GC-MS results of sample Xp20

No	RT	Formula	Area%	Molecular	Name
1	5.323	$C_6H_{12}O_2$	3.96	116.158	4-hydroxy-4-methylpentan-2-one
2	49.074	$C_{18}H_{20}$	0.44	236.351	[(*E*)-2-methyl-4-phenylpent-3-en-2-yl]benzene
3	66,419	$C_{14}H_{28}O$	0.81	212.370	1-ethenoxy-2,6,8-trimethylnonane
4	81.146	$C_{24}H_{38}O_4$	18.93	390.556	bis(2-ethylhexyl) benzene-1,2-dicarboxylate
5	85.930	C_{16} H_{12}	71.75	284.268	1,8-Dihydroxy-3-methoxy-6-methyl-9,10-
6	98.101	$C_{18}H_{34}O_2$	4.11	282.461	Ethenyl hexadecanoate

*: RT: Retention time, min: minute, Area %: Peak area %

Table 3 shows that the Xp20 sample contained 1,8-Dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone at the highest value of 71.25% than other samples. This is consistent with the spectrum result.

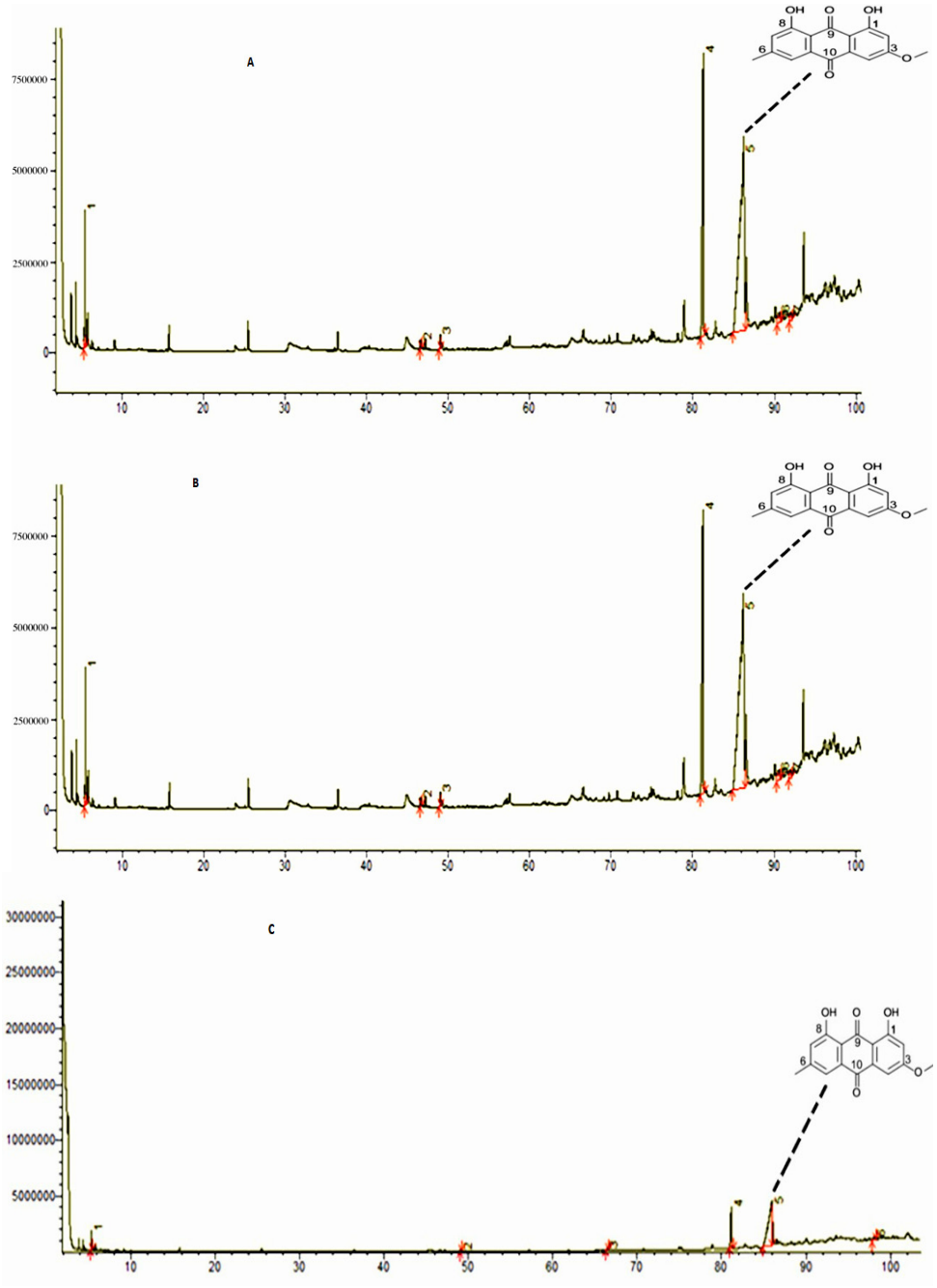

Figure 1. GC-MS spectrum of lichen samples; A: Xp3, B: Xp14, C: Xp20

Figure 1 shows the spectra containing the GC-Mass analysis results of three different lichen samples. The highest peak in each of these shows that the common anthraquinone structures are at the highest rate in each of them.

Total phenolic and flavonoid content results

TPC and TFC of the three extracts had assessed spectroscopically (Table 4). TPC of the extracts ranged between 17.07 and 25.30 mg GAE g^{-1}, (R^2=0.999). The highest TPC was obtained for extract Xp20 while the lowest TPC was obtained for extract Xp14. Quercetin was standard for TFC. The results are summarized in Table 4. The highest TFC was obtained for extract Xp20 (51.42±0.15 mg QE. g^{-1}) while the lowest TFC was obtained for extract Xp14 (23.73±0.18 mg QE. g^{-1}).

Table 4. The total phenolic and total flavonoid contents of the Xp extracts

Extracts	Total Phenolic Content mg GAE/g	Total Flavonoid Content mg QE/g
Xp3	18.27±0.10	28.35±0.25
Xp14	17.07±0.11	23.73±0.18
Xp 20	25.30±0.05	51.42±0.15

DNA Binding results

The DNA binding properties of the studied extracts are showed in Figure 2. Lanes 1-3 contained different concentrations of CT-DNA in the absence of the extracts. In the presence of the extracts (lanes 4-12), as the CT-DNA concentration decreased the smear light intensity increased. The highest CT-DNA intercalation activity was obtained for sample Xp20 (lanes 10 and 11).

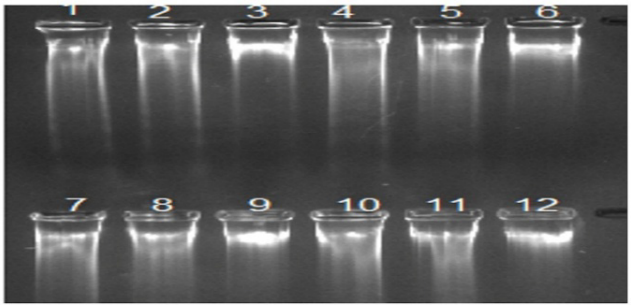

Figure 2. DNA Binding activity of Xp3, Xp14, and Xp20 extracts. Lane (1) CT-DNA (2 mg/ mL) + Buffer. Lane (2) CT-DNA (1 mg/mL) + Buffer. Lane (3) CT-DNA (0.5 mg/ mL) + Buffer. Lane (4) CT-DNA (2mg/ mL) + Buffer + 1.0 % Xp3. Lane (5) CT-DNA (1 mg/ mL) + 1.0 % Xp3+ Buffer. Lane (6) CT-DNA (0.5 mg/ mL) +1.0 % Xp3+ Buffer. Lane (7) CT-DNA (2mg/ mL) + Buffer + 1.0 % Xp14. Lane (8) CT-DNA (1 mg/ mL) + Buffer + 1.0 % Xp14. Lane (9) CT-DNA (0.5 mg/ mL) + Buffer + 1.0 % Xp14. Lane (10) CT-DNA (2 mg/mL) + Buffer + 1.0 % Xp20. Lane (11) CT-DNA (1 mg/mL) + Buffer + 1.0 % Xp20. Lane (12) CT-DNA (0.5mg/mL) + Buffer + 1.0 % Xp20 (Ladder dye was put into every lane).

DNA Cleavage results

DNA cleavage properties of the studied extract are shown in Figure 3. The same results were obtained as there was no cleavage in lanes 1 and 7. Lanes 2, 8 and 11 did not contain DNA, so the samples did not emit with UV light. Acetone did not affect polar DNA (lane 5), whereas methanol, being a polar solvent, affected DNA (lane 6). There was no significant cleavage in lanes 3 and 4 containing sample Xp3 compared to lane 1. However, weak cleavage was seen in lane 9 in the presence of sample Xp14. Furthermore, thicker Form II and thinner Form I occurred in lanes 12 and 13 containing Xp20, resulting in DNA cleavage (over 50 percent).

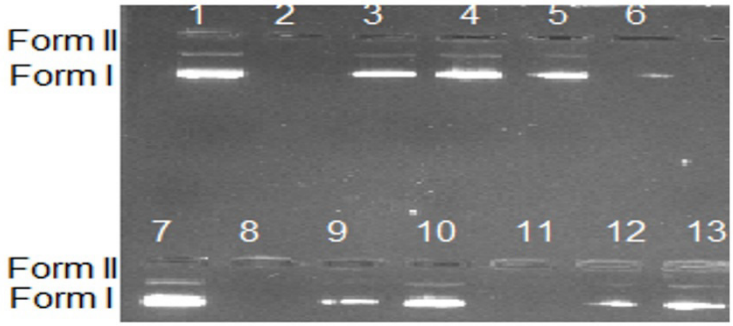

Figure 3. DNA Cleavage activity in 1% agarose gel of Xp3, Xp14, Xp20 extracts. Lane (1) pBR322DNA + ddw. Lane (2) Xp3+ ddw. Lane (3) pBR 322DNA + 1% Xp3+ddw. Lane (4) pBR 322DNA + 0.5% Xp3+ddw. Lane (5) pBR 322DNA + Aceton. Lane (6) pBR 322DNA + Methanol. Lane (7) pBR 322DNA + ddw. Lane (8) Xp14+ ddw. Lane (9) pBR 322DNA + 1% Xp14+ddw. Lane (10) pBR 322DNA + 0.5% Xp14+ddw. Lane (11) Xp20+ ddw. Lane (12) pBR 322DNA + 1% Xp20 +ddw. Lane (13) pBR 322DNA + 0.5% Xp20 +ddw (Ladder dye was put into the every lane).

Cytotoxicity results

The control group is the negative control group. In other words, it is the microscopic observation of the culture medium containing only the cells without any added substance. Therefore, since there is no additional substance medium that will disrupt the structure of the cells in this group, the cells are seen in the main form with the highest column in maximum viability numbers in Figure 4. During cultivation, the cells that incubated with Xp14 extract had abnormal morphology, cells were demonstrated shrinkage structure. For this study, it was tested by using MTT cell proliferation test *in vitro* cytotoxic effects of compounds on DLD-1 cell line. The obtained results of cytotoxic effects were presented Figure 4.

Some concentrations of lichen samples continued to show cell proliferation without causing cell toxicity. According to Figure 4, Xp14 extract reduced cell proliferation with the increasing concentration. On the other hand, cell proliferation decreased as the concentration of Xp20 and Xp3 extracts.

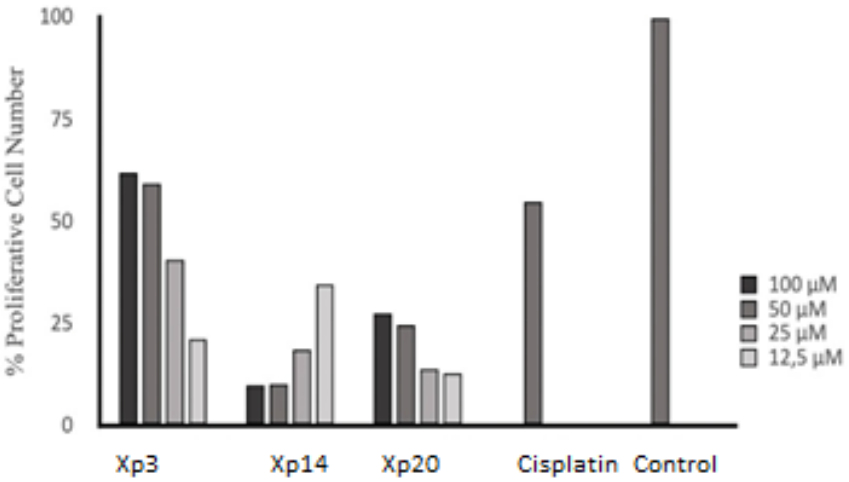

Figure 4. Cell proliferation at different concentrations of lichen extract (12.5 µM, 25 µM, 50 µM, and 100 µM), positive control (cisplatin,10 µM), and negative control (without extract)" on the DLD-1 cell line experiments

DISCUSSION

Xp samples were taken from different cities located in different regions of Turkey and their chemicals were quantitatively analyzed. This study indicated that sample Xp20 collected from the province of Kütahya, located in the Central West Anatolian part of the Aegean region, contained more phenolic, flavonoid, and anthraquinone derivatives. In Tables 1-3, which were created according to the GC-MS results performed on the *Xanthoria parietina* (Xp) contents used in this study, it was seen that 1,8-dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone structure was more prevalent in the 50-71% range. This achievement can originate from the fact that the Aegean region has maquis vegetation and a Mediterranean climate [36]. Anthracene (Parietin) is a carcinogenic organic substance since it contains polycyclic aromatic hydrocarbons [37-39]. However, this compound and its derivatives are used as a dyestuff source in the food, medicine, and textile industries [40-42]. Since Xp 20 contains the highest amount of anthraquinone group at 71.75%, it can be used as a dye source in

the textile and food industries. The study of the DNA cleavage and DNA binding activities of materials is a suitable method to examine their anticancer properties [43-44]. Therefore, the DNA properties and toxicity of the parietin structures were investigated in this study. In the cytotoxicity evaluation of three Xp extracts at 100μM concentration, that is, in their most concentrated state, the closest cell proliferation to control and cisplatin was Xp 3, then Xp 20. In the study conducted by diluting these, cell proliferation continued to increase as Xp 14 did not show a toxic effect. However, since Xp 3 and Xp 20 reduce the number of cancer cells with a toxic effect, they are seen to have anticancer potential.

Marian Temina et al carried out GC-MS and HPLC analysis for the determination of acids and esters in the lichen-type structures of Collema [45]. The phenolic-flavonoid phytochemical content of Xanthoria species growing in Türkiye is higher than other Rocella lichen species.[46]. Torres et al. studied the surface alkanes and fatty acids content of *Xanthoria parietina* lichen collected from the Jerusalem hills using the GC-MS method [47]. Basile et al. reported the parietin content of *Xanthoria parietina* by performing the HPLC method. The results proved that the pure parietin extracts as secondary metabolites exhibited effective antimicrobial and anticancer properties [34,35;47]. Also, phytochemical features of *Xanthoria parietina* lichen obtained from Australia were studied only using spectroscopic methods. In this study, the phenolic-flavonoid content of Roccella lichen species was studied by applying the same spectrophotometric method [48,49]. No DNA cleavage or DNA binding studies have been found for the *Xanthoria parietina* species in the literature. In this study, DNA cleavage was significant only in Xp 20, while it was very weak in the others. More biological activities such as cytotoxicity and significant DNA cleavage were observed in Xp samples with high anthraquinone content. Solutions of Xp3 and Xp20 Lichen samples were prepared as 100 μM. However, it is seen in Figure 4 that the samples showed a cytotoxic effect in the cytotoxicity test applied at a lower concentration of 12.5 μM and reduced cancer cell proliferation. On the other hand, the Xp14 lichen sample showed the opposite activity at all concentrations, i.e., it did not show a cytotoxic effect. It has been determined that cytotoxicity is also lowest in Xp 14 sample with low flavonoid, phenolic or anthraquinone content.

CONCLUSION

This study presents new *Xanthoria parietina* samples, which include mostly parietin/flavonoid/phenolic content and were obtained from different regions of Turkey for the first time. Due to 71.75% anthracene (parietin) and high

flavonoid-phenolic contents, the *Xanthoria parietina* lichen (Xp20) collected from Kütahya city of Turkey can be used as a natural dyestuff source. Since Xp3 (from Yozgat) and Xp 20 (from Kütahya) Lichen samples show cytotoxic effects at low concentrations such as 12.5 µM, they can be evaluated as anticancer drugs and chemotherapy agents when supported by additional studies.

MATERIALS AND METHODS

Lichen Samples

Samples of *Xanthoria parietina* species (Figure 5) were harvested properly from the wild as described below and transferred to the laboratory within 6 hours. Lichenological identities of lichen materials were carried out at Biology Department Herbarium (ERC), Science Faculty, Erciyes University, Kayseri, Turkey. The voucher specimens were deposited at ERC. The herbarium information of the samples is detailed as follows:

Xanthoria parietina (Xp3) Th.Fr.; Yozgat, Çamlık; on Fagus orientalis, GPS coordinates 39°48'52.98'' N, 34°48'48.81''E, 1375 m, Turkey, February 2011, Herbarium code: ERC-Xp3; Collector: Mehmet Gökhan Halıcı, Emre Kılıç.

Xanthoria parietina (Xp14) Th. Fr.; İzmit, Kandıra; east of Cebeci, frutices in litore. GPS coordinates 41°12' 04'' N, 30°15'46''E, 10 m, Turkey, May 2012, Herbarium code: ERC-Xp14; Collector: Mehmet Gökhan Halıcı, Emre Kılıç.

Xanthoria parietina (Xp20) Th. Fr.; Kütahya, between Kütahya and Afyon, northeast of Körs village, Salix communities. GPS coordinates 39°19'10'' N, 30°17'16''E, 1095 m, Turkey, June 2012, Herbarium code: ERC-Xp20; Collector: Mehmet Gökhan Halıcı, Emre Kılıç.

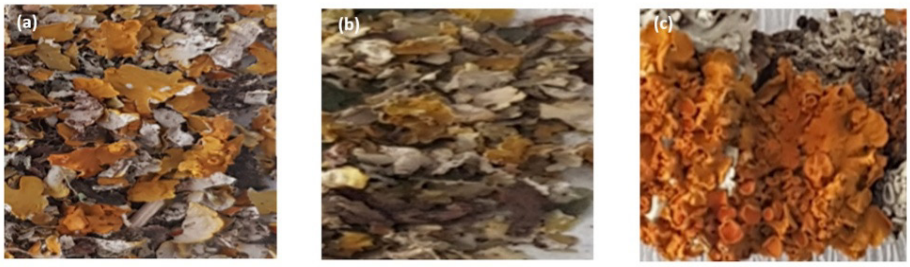

Figure 5. Images of the collected *Xanthoria parietina*:
(a) Xp3, (b) Xp14, (c) Xp20

Chemicals and Other Materials

Methanol, Ethanol, acetone, DMSO, TAE and Folin Ciocalteau, sodium carbonate, aluminium nitrate, potassium acetate, sodium hydroxide, ethidium bromide, Tris HCl were supplied by Merck (Darmstadt, Germany). CT-DNA, 1% Agarose, glycerol, FBS, penicilline-streptomycin got from Sigma-Aldrich (St. Louis, MO, USA). pBR322 plasmid DNA was used DNA model for cleavage activity by Thermo Fisher Scientific (Baltics UAB | V.A. Graiciuno 8, LT-02241 Vilnius, Lithuania).

Preparation of the extracts from *Xanthoria parietina* samples

Soxhalation was used for the preparation of solvent extracts. A 1.00 g of shade-dried Xp samples were taken separately in a thimble and the extracts were collected using 20 ml acetone. Then, the filtrates were concentrated by a rotary evaporator (BuchiR200 Rota vapor). The extracts resuspended in DMSO and kept at 4°C in a refrigerator for further use [48-49].

Gas Chromatography-Mass Analysis Procedure

GC-MS analysis of the extracts was performed on a SHIMADZU QP2010 ULTRA GC System fitted with a Rtx-5MS capillary column (30 m 0.25 mm inner diameter, 0.25 µm film thickness, max. temperature, 350 °C) coupled to a SHIMADZU GC-MS. Pure, ultra-high helium (99.99%) was used at a sustained flow rate of 1.0 mL/ min. Ion source temperatures and transfer line injection were all 290 °C. The ionizing energy was 70 eV. The electron multiplier voltage was obtained from an auto-tuning. The oven temperature was programmed from 60°C (hold for 2 minutes) to 280°C at a rate of 3°C/min. The samples were diluted with a convenient solution (1/100, v/v) and filtered. The particle-free diluted extracts (1 µL) were aspirated into a syringe and injected into the injector at a split ratio of 50:1. All data were obtained from the full-scan mass spectra within the scan range of 40-850 amu. The percentage composition of the sample extracts was expressed as a percentage by peak area. The characterization and identification of the chemical compounds in various sample extracts were based on the GC retention time. The mass spectra were computer-matched with those of standards available in mass spectrum libraries.

Determination of Total Phenolic Content (TPC)

Total phenolic content was determined using the Folin–Ciocalteu colorimetric method. Gallic was used as standards. Briefly, 20 µL of the filtered extracts were mixed with 400 µL of 0.5 N Folin-Ciocalteu reagent and 680 µL distilled water. This mixture was incubated for 3 min at room temperature before adding 400 µL Na_2CO_3 (10%). After incubation of the samples for 2 hours,

their absorbances were measured at 760 nm with the UV-Vis machine (Thermo Multiskan Go). The concentration of total phenolic compounds was calculated as mg gallic acid equivalents (GAE) per g dried extract [50].

Determination of Total Flavonoid Content (TFC)

The aluminum complexation method was used to determine the total flavonoid content. 0.5 mL of plant extract, 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M potassium acetate, and 4.3 mL of 80% ethyl alcohol were combined using this method. The samples were incubated at room temperature for 40 minutes, and then their absorbance at 415 nm was measured using a UV-Vis device (Labomed Inc., Culver City, USA). Using quercetin as the standard, a curve for calibration in the range of 0.00195 to 0.5 $mg.mL^{-1}$ ($r^2 = 0.999$) was created. Based on the average of three measurements, the total flavonoid concentration was expressed as mg of quercetin equivalent (QE) per g of dry weight (dw) [51].

DNA Cleavage Test

The DNA cleavage properties of the Xp3, Xp14, Xp20 extracts that the process was carried out in 1% agarose gel electrophoresis device. No light was used for interaction prior to sample and DNA incubation. Supercoiled pBR322 plasmid DNA had used with a decreasing percentage of the extracts (1%, 0.5%) in ddw and Tris HCl buffer (pH=7). Gel electrophoresis process was applied in the referenced study with a few changes [52].

DNA Binding Test

The samples were prepared by the dissolution of Xp3, Xp14, and Xp20 extracts in ddw. The different concentrations of Calf Thymus DNA (CT-DNA) (2-0.5mg/ml) in Tris HCl buffer and a constant concentration of the extracts (1%). The mixtures were adjusted to a final volume of 25µL with buffer and incubated at 37°C for 24 hours. Then, the mixtures were loaded on 1% agarose gel with ethidium bromide staining in Tris Acetate Edta (TAE). The electrophoresis was carried out at 80 V for 45 minutes. The results were visualized using the BioRad Gel Doc XR system [53].

Cytotoxicity Test

Human colon cancer cell line (DLD-1) for this study was obtained from American Type Culture Collection CCL-221™, ATCC, USA). DLD-1 cells in nitrogen tank were dissolved at 37°C for one minute. Dissolved cells were placed in a falcon tube, FBS was added to remove DMSO, and pipetting was done several times. Then, RPMI-1640 (Sigma) cell medium containing 10% Fetal bovine serum (FBS) and 1% penicillin-streptomycin was used.

Cells were cultureted in 75 cm^3 flasks with RPMI-1640 medium. 5 % CO_2 and 37 ˚C humidified incubator was used. Via Cells were seeded as $5x10^3$ cells per well in 96 well plate for MTT assay. The extracts were added separately to the cells as 100 (1%), 50 (0.5%), 25 (0.25%), 12.5µM final concentrations after 24 hours after seeding. 10 µM Cisplatin was used as a positive control. There is no lichen extract in the positive control, but the anticancer drug cisplatin is present. There is no lichen extract in the negative control.All groups were incubated at % 5 CO_2 and 37 ˚C humidified incubator for 24 hours. After the medium liquids of the incubated cells were withdrawn. The remaining process was completed as in the reference. [54-55].

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AUTHORS CONTRIBUTIONS

NG: Conception, design, data collection and analysis, manuscript writing and editing, and supervision. EKS: Material preparation, data collection and analysis, manuscript writing and editing.

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The author did not receive support from any organization for the submitted work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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