TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2017) 41: 484-493
© TÜBİTAK
doi:10.3906/biy-1609-40

Changes in apoptosis-related gene expression profiles in cancer cell lines exposed to usnic acid lichen secondary metabolite

Adnan Berk DİNÇSOY[1,2], Demet CANSARAN DUMAN[1,*]
[1]Biotechnology Institute, Ankara University, Ankara, Turkey
[2]Department of Physiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey

Received: 19.09.2016 • **Accepted/Published Online:** 12.01.2017 • **Final Version:** 14.06.2017

Abstract: The presence of uninhibited side effects of cancer drugs often used in cancer treatment has stimulated the search for alternative therapeutic approaches. Therefore, anticarcinogenic effects of synthetic, herbal, and fungal drugs have been investigated for the treatment of various cancer types in recent studies. Lichens, symbiotic organisms of fungi and algae, synthesize metabolites with significant biological activities. The aim of the current study was to screen the anticancer potential of usnic acid on various types of nonmalignant cell lines (Vero, L929) and cancer cell lines (CaCo2, RD, Hep2C, HepG2, Wehi). The growth inhibitory effect of usnic acid was determined by MTT assay. Since this study was also designed to explore mRNA expression profiles, this paper is the first to look into the effects of usnic acid on apoptotic gene expression. The effects of usnic acid on the gene expression patterns of the tumor suppressor gene *p53*, proapoptotic gene *Bcl-2*, and *Bax* were studied with qRT-PCR. There was an approximately ninefold decrease in the *p53* and *Bcl-2* expression for usnic acid in the Wehi cancer cell line. Consequently, it is concluded that usnic acid has tumor inhibitory properties, and if indicated by further works like animal studies and clinical trials, it may be used therapeutically in the future.

Key words: Apoptosis, cytotoxicity, qRT-PCR, usnic acid

1. Introduction

Cancer, which is a major disease caused by uncontrolled cell growth (Hanahan and Weinberg, 2011), can be treated by methods like surgery, radiation therapy, and chemotherapy. However, these therapeutic methods have some unwanted side effects, including the exposure of nonmalignant cells to cytotoxic agents (Kim et al., 2015).

Natural products are potentially remarkable sources as new drugs in cancer treatment (Stanojkovic, 2015). Some researchers have recently screened the effects of natural metabolites obtained from different biological organisms on cancer cells. Among them, some have been used in the pharmaceutical industry. Studies conducted on many natural metabolites have resulted in the production of pharmaceutically significant drugs, and many potential sources of drug therapies still need to be investigated (Saleem et al., 2010). Approximately 60% of these drugs are directly or indirectly made up of natural products (Newman, 2008), one of which is lichens. Lichens, symbiotic organisms of fungi and algae, synthesize low-molecular-weight secondary compounds with significant biological activities and have shown intense antibiotic, antiproliferative, antioxidant, anti-HIV, anticancer, immunomodulator, and antiprotozoal activities.

Lichens are known to synthesize more than 1000 various secondary metabolites produced by the fungal partner. Although lichen metabolites demonstrate biological roles, one of their main disadvantages is their slow growth rate in laboratory conditions. Therefore, lichens have received little attention in the biomedical field. However, presently, lichens are more successfully cultured in laboratory settings thanks to advances in technology (Yamamoto et al., 1985, 1987, 1993; Stocker-Wörgötter, 2001; Stocker-Wörgötter and Elix, 2002; Behera et al., 2006). Thus, lichen species and their related metabolites can now be used in the large volumes necessary for commercial exploitation in the pharmaceutical field.

As a result of some studies, lichens and their secondary metabolites have demonstrated cell proliferation by regulating the cell cycle and an increment in cell death by inducing apoptosis or necrosis (Nguyen et al., 2014; Ari et al., 2014; Coskun et al., 2015; Kim et al., 2015; Kılıç et al., 2016). The mechanism of many anticancer drugs in the pharmaceutical industry is based on their ability to induce apoptosis (Motomura et al., 2008; Florea and Busselberg, 2011). Apoptosis that occurs through different ways can be enhanced in cells. First, candidate molecules suppress the proliferation of malignant cells, and apoptosis

* Correspondence: dcansaran@yahoo.com

increases thereafter and shows a mechanistic approach to cancer chemoprevention and chemotherapy (Alhazmi et al., 2014). In an article by Singh et al. published in 2013, the cell death ratio of human lung carcinoma was determined after 25–100 μM usnic acid application for 24 and 48 h. They observed that usnic acid induced mitochondrial membrane depolarization and showed approximately twofold change in apoptotic cells as regards flow cytometry analyses (Singh et al., 2013). Zuo et al. explored the antitumoral activity of usnic acid in human breast cancer MCF-7 by in vitro and in vivo experiments. They demonstrated that usnic acid induces apoptosis of MCF-7 cells by reactive oxygen species generation, c-Jun amino-terminal kinases activation, mitochondrial dysfunction, cytochrome-c release, and caspase activation (Zuo et al., 2015). Nguyen et al. showed the cytotoxic activity of 17 lichen species on some human cancer cells and determined molecular mechanisms of their anticancer activity. The results of their study revealed that secondary metabolites extracted from *Flavocetraria cucullata* show significant anticancer effects on cancer cell lines through the induction of apoptosis and suppression of tumorigenic potentials (Nguyen et al., 2014).

Usnic acid, a lichen secondary metabolite, has been extensively studied since its first isolation in 1844. Its chemical formula is 2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3 (2H, 9bh). The mechanism of usnic acid needs to be investigated in greater detail to reach clinical trials. Usnic acid has been mainly investigated for cell proliferation; however, no research has been conducted to look into the effect of usnic acid on cancer cell lines for the molecular mechanism of apoptosis at the mRNA level. Therefore, this study was designed to determine the effect of usnic acid-induced apoptosis in the examined cancer cell lines by apoptosis-related gene expression. In order to test this, we determined the role of usnic acid in inhibiting cell growth and modulating the expression of the *p53*, *Bax*, and *Bcl-2* genes.

2. Materials and methods

2.1. Lichen samples

Samples of *Usnea diffracta* were collected from the mountainous forest region in Trabzon-Uzungöl-Soğanlı, Turkey (44°84′N, 37°61′E, 1799 m) by the second author. Identification of the collected samples was done according to the keys of Wirth (1995) and Purvis et al. (1992). Fresh samples were stored at 4 °C until analysis. Commercial usnic acid was purchased from Sigma and 400 μM usnic acid, which is the stock concentration, was prepared in 14 mM dimethyl sulfoxide (DMSO). Appropriate dilutions of this stock solution (200, 100, 50, 25, 12.5, and 6.25 μM) were made with DMSO.

2.1.1. Isolation and identification of usnic acid

Air-dried *Usnea diffracta* lichens were extracted with 512 g of light petroleum at low temperature (2–4 °C). Afterwards, the sample was recrystallized ten times with pure ethanol and 93.6 mg of usnic acid was obtained. The usnic acid extract was incubated in darkness until high-performance liquid chromatography (HPLC) analysis was conducted. Before the experiments commenced, the extract was filtered through a 0.45-μm filter, and 20 μL of extract was analyzed by being injected into the HPLC system.

Usnic acid (1 mg/mL) was prepared in acetone and used as a stock solution. An appropriate further dilution was made with acetone. All standards were placed in an autosampler and analyzed. Calibration curves were prepared with seven various usnic acid concentrations for linear regression analysis. A Thermo Finnigan HPLC system equipped with a Surveyor LC pump, Surveyor photodiode array detector, Surveyor autosampler, and data processor (ChromQuest 4.01) was used. HPLC analysis of usnic acid was performed according to the protocol of Cansaran et al. (2007). Each analysis was carried out in triplicate.

2.2. Cell culture

Human colorectal adenocarcinoma cell line CaCo2, human hepatocellular carcinoma cell line HepG2, human (HeLa derivative) cervix carcinoma cell line Hep2C, human rhabdomyosarcoma cell line RD, mouse fibrosarcoma cell line Wehi, mouse subcutaneous connective tissue cell line L929, and African green monkey kidney cell line Vero were obtained from Animal Cell Culture Collection of the Foot and Mouth Disease Institute, Turkey. CaCo2, HepG2, Hep2C, RD, L929, and Vero cells were cultured in Dulbecco's modified Eagle medium (PAA Laboratories, Austria); Wehi was cultured in RPMI-1640 containing 10% fetal bovine serum (Gemini Byproducts, USA), 2 mM L-glutamine, and 1% penicillin and streptomycin. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2.

2.3. Cell viability assay

CaCo2, HepG2, Hep2C, RD, Wehi, L929, and Vero cells were plated into a 96-well culture plate at a density of 1×10^4 cells per well. Commercial usnic acid and natural usnic acid were then diluted to appropriate concentrations and immediately applied to the cells. Concentration- and time-dependent cytotoxicity were assessed by having the cells exposed to usnic acid at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 μM for 24, 48, and 72 h. Viability of the cells was evaluated using the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. First, 50 μL of MTT was added to the medium for 4 h of incubation. The insoluble formazan crystals within the cells were extracted by 100 μL of isopropanol, and the absorbance was measured using a microplate reader at a

wavelength of 570 nm. All experiments were performed in six independent biological and technical replicates. EC_{50} values and survival rates were calculated by obtained absorbance values. Absorbance values were converted to a logarithm base for the calculation of EC_{50} values, and the calculations were then performed using GraphPad Prism 6.

2.4. Total RNA extraction and cDNA synthesis

Examined cells (5×10^4 cells/well) were cultured in 6-well plates. Cells were treated with EC_{50} concentrations of usnic acid for 48 h. After the incubation period, the culture medium was aspirated, and total RNA was isolated from the cultured cells using a TRIzol reagent (GENEzol, Geneaid Biotech, Taiwan) according to the manufacturer's protocol. Briefly, the examined cells were harvested for RNA extraction by centrifugation, the supernatants were discarded, and 500 µL of TRIzol was added to the cells and homogenized for 1 min. Chloroform (100 µL) was then added and vortexed for 15 s. Samples were incubated for 3 min at room temperature and centrifuged for 15 min at 14,000 rpm (4 °C), and the supernatants were transferred to new tubes. An equal volume of isopropanol was added, incubated for 10 min at room temperature, and then centrifuged for 10 min at 14,000 rpm (4 °C). The supernatants were discarded, and 800 µL of 75% ethanol was added to the pellet, followed by centrifugation for 5 min at 13,200 rpm (4 °C). The supernatants were discarded, and the pellets were suspended in 20 µL of DEPC-water and treated with RNase-free DNase I (Takara, China). Purity and concentration of the total RNA were determined by spectrophotometry using the 260 nm/280 nm ratio (NanoDrop 1000, Thermo Scientific, USA) and 1% agarose gel electrophoresis. First-strand cDNA was synthesized based on the total RNA of each sample using the Roche First Strand cDNA Synthesis Kit (Roche, Switzerland) according to the manufacturer's protocol. cDNA synthesis was applied with 2 µg of RNA, 2.5 µM anchored-oligo $(dT)_{18}$, 1X Transcriptor High Fidelity Reverse Transcriptase reaction buffer, 20 U of protector RNase inhibitor, 1 mM deoxynucleotide mix, and 10 U Transcriptor High Fidelity Reverse Transcriptase at the final concentration. Afterwards, it was continued at 10 min at 65 °C, 30 min at 55 °C, and 5 min at 85 °C.

2.5. Real-time PCR analysis

Real-time quantitative PCR (qRT-PCR) was conducted with the Roche Light Cycler 480 Real Time, using SYBR Green I Master. Sequences of tumor suppressor gene *p53*, antiapoptotic gene *Bcl-2*, and the *Bax* gene were designed with the Primer3 program (Table). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. All experiments were performed in three independent biological and technical triplicates. Melting curve analysis was performed to determine the effectiveness of PCR and to observe if there was any dimer formation. *p53*, *Bcl-2*, *Bax*, and the reference gene (*GAPDH*) were optimized by the testing of real-time PCR reactions. Reaction conditions after predenaturation were studied at 10 min at 95 °C and 40 cycles of 10 s at 95 °C, 30 s at 60 °C, and 8 s at 72 °C.

2.6. Statistical analysis

The relative mRNA expression profile of the three targeted genes was normalized with the reference gene (*GAPDH*) and calculated by $2^{-\Delta\Delta Ct}$ method. Statistical analyses were conducted with GraphPad Prism software (Version 5.0, San Diego, CA, USA) and one-way ANOVA with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Fisher's least significant difference test at a P-value of 0.05 significance was applied.

3. Results

3.1. Identification of usnic acid

Quantitative analysis of usnic acid in *Usnea diffracta* species was performed with HPLC. Identification of peaks in chromatograms of lichen extracts was attained with the comparison of retention times with that of standard usnic acid. A sample representing these chromatograms is shown in Figure 1. HPLC retention time was 12.22 min at 1.6 mL min^{-1} flow rate.

Table. Primer sequences of the examined genes.

Primers	Sequence (5'→3')	Product length (Pl)	Annealing temperature (Tm)
P53	5' AACGGTACTCCGCCACC 3' 5' CGTGTCACCGTCGTGGA 3'	94	60.0 °C
Bcl-2	5' CTGCACCTGACGCCCTTCACC 3' 5' CACATGACCCCACCGAACTCAAAGA 3'	119	65.0 °C
Bax	5' TCCCCCCGAGAGGTCTTTT 3' 5' CGGCCCCAGTTGAAGTTG 3'	68	65.0 °C
GAPDH	5' AACGGGAAGCTTGTCATCAATGGAAA 3' 5' GCATCAGCAGAGGGGGCAGAG 3'	194	60.0 °C

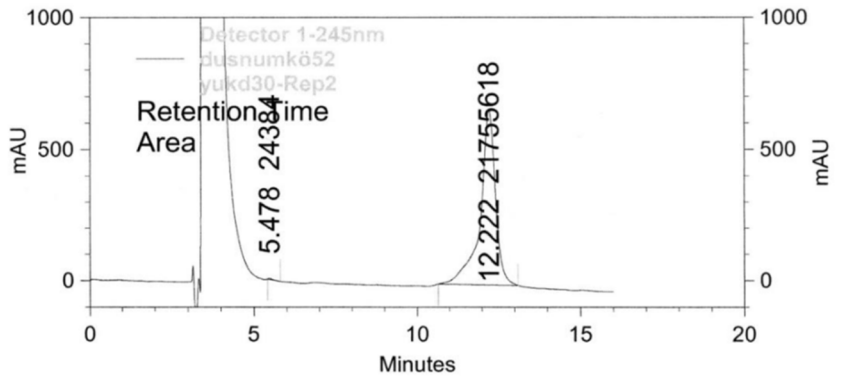

Figure 1. A sample chromatogram for the analysis of usnic acid from *Usnea diffracta* by HPLC [solvent (tR = 5.4 min); usnic acid (tR = 12.22 min)].

3.2. Cell death

The testing of natural and commercial usnic acid secondary metabolites on various cell lines proved a concentration- and time-dependent action as evaluated by the MTT cytotoxicity assay. According to the results, natural and commercial usnic acids had similar activity ratios against all nonmalignant and cancer cell lines. There was no significant result of either usnic acid secondary metabolite. Cell viability of Hep2C cancer cells exposed to natural and commercial usnic acids for 48 h is given in Figure 2. Reduction in RD cancer cell viability with usnic acid treatment at a concentration of 6.25 µM to 25 µM after 24 h ranged from 2.1% to 27.2%, whereas after 48 and 72 h, it respectively ranged from 31.3% to 69.1% and 57.7% to 85.9%, as shown in Figure 3. It should be noted that the 6.25 and 25 µM concentrations of usnic acid treatments after 48 h were found to show significant cytotoxic effects on the examined cancer cell lines ($P < 0.05$) (Figure 3). Additionally, it was found in our study that usnic acid, which had a cell viability-reducing effect on all cancer cells, was most effective on the RD and Wehi cancer cell lines ($P < 0.05$). This result suggests that usnic acid inhibited the proliferation of the examined cancer lines. It is surprising that there was no significant decrease in cell viability of the L929 and Vero cell lines at the increasing concentrations/times of usnic acid application, as shown in Figure 4 ($P < 0.05$). One of the most important results of the current study is that usnic

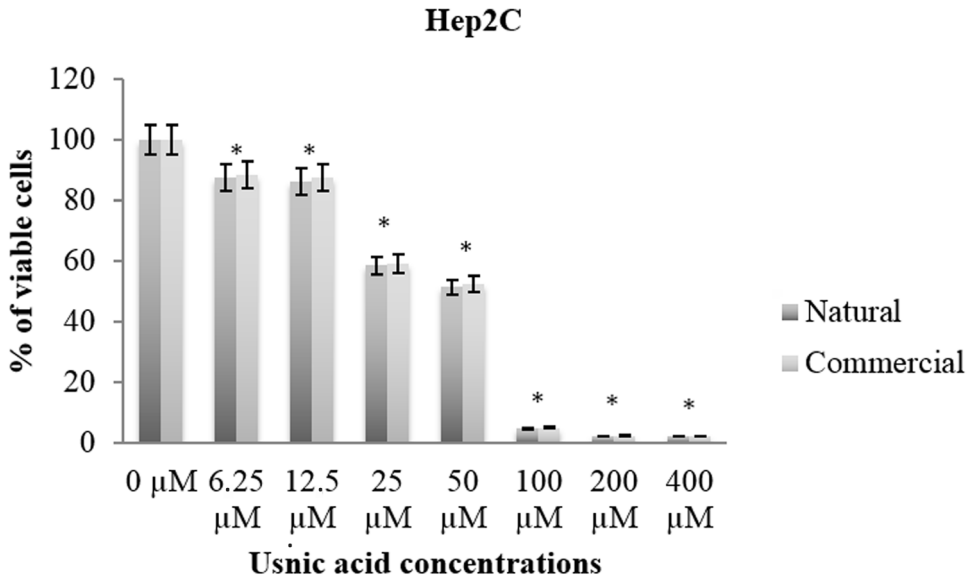

Figure 2. Dose-response curves for the effect of natural and commercial usnic acid on Hep2C cell line for 48 h (values are statistically significant at *$P < 0.05$ compared to the respective control).

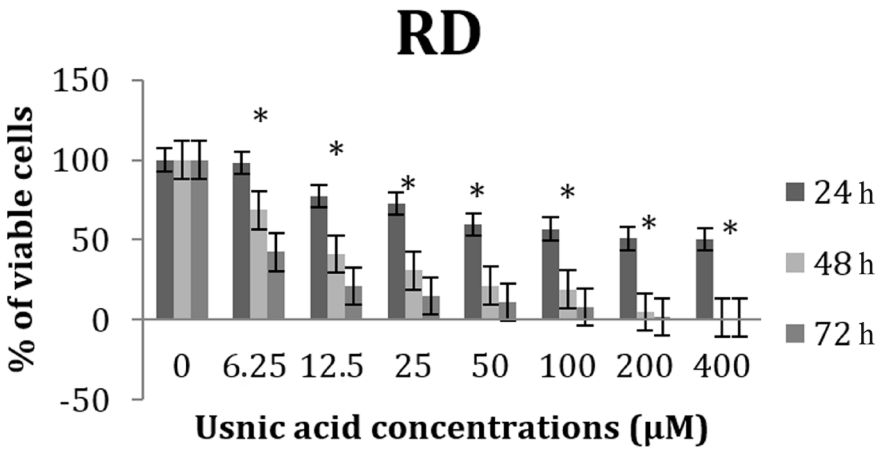

Figure 3. Comparative representation of the RD cell line (values are statistically significant at *P < 0.05 compared to the respective control).

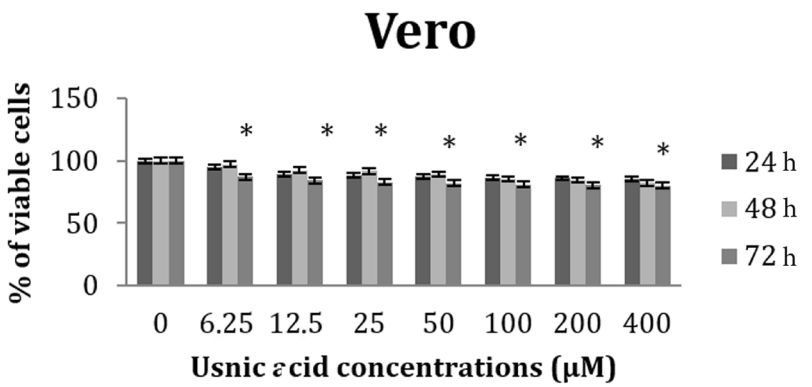

Figure 4. Comparative representation of the Vero cell line (values are statistically significant at *P < 0.05 compared to the respective control).

acid inhibited cancer lines but had very little inhibiting effect on nonmalignant cell lines. Particularly, cancer can be treated by chemotherapy, and this type of therapy uses chemical compounds that inhibit cytotoxicity in cancer cells. As a consequence, the application of chemotherapy may cause numerous unwanted side effects. In order to overcome these negative side effects, usnic acid is found to be a promising source for cancer treatment.

Vast differences among the examined cell lines as well as different sensitivities to usnic acid secondary metabolites were shown by calculating the half maximal effective concentrations (EC_{50}). EC_{50} values for usnic acid on the CaCo2, HepG2, Hep2C, RD, and Wehi cancer cell lines were 7.05, 15.4, 21.8, 22.9, and 15.8 µM, respectively.

3.3. Gene expression analysis

Commercial usnic acid was selected in our gene expression studies since cytotoxic effects of commercial and natural usnic acids were similar. After having exposed the tested cell lines to usnic acid doses corresponding to EC_{50} values for 48 h, total RNAs were successfully isolated from all cell lines except the CaCo2 and HepG2 cell lines. Therefore, qRT-PCR results of the CaCo2 and HepG2 cell lines were not included in this study. RNA concentrations were in the range of 157 to 261 ng/µL, and 230/260 nm ratios were between 0.87 and 0.99. The integrity of the extracted RNAs was also evaluated by electrophoresis. So as to investigate the effects of usnic acid on molecular mechanisms related to apoptosis in cancer cell lines, the expression of some apoptosis related genes (*Bax*, *Bcl-2*, and *p53*) was analyzed by qRT-PCR. There are no available data on the effect of usnic acid on proapoptotic and antiapoptotic gene expression in different cancer and nonmalignant cell lines. Therefore, qRT-PCR assay of some apoptosis-related genes in the cell lines after exposure to usnic acid is one of the valuable points of the current study. Transcription changes of the examined genes subjected to EC_{50} doses of usnic acid treatments are given in Figures 5a–5d.

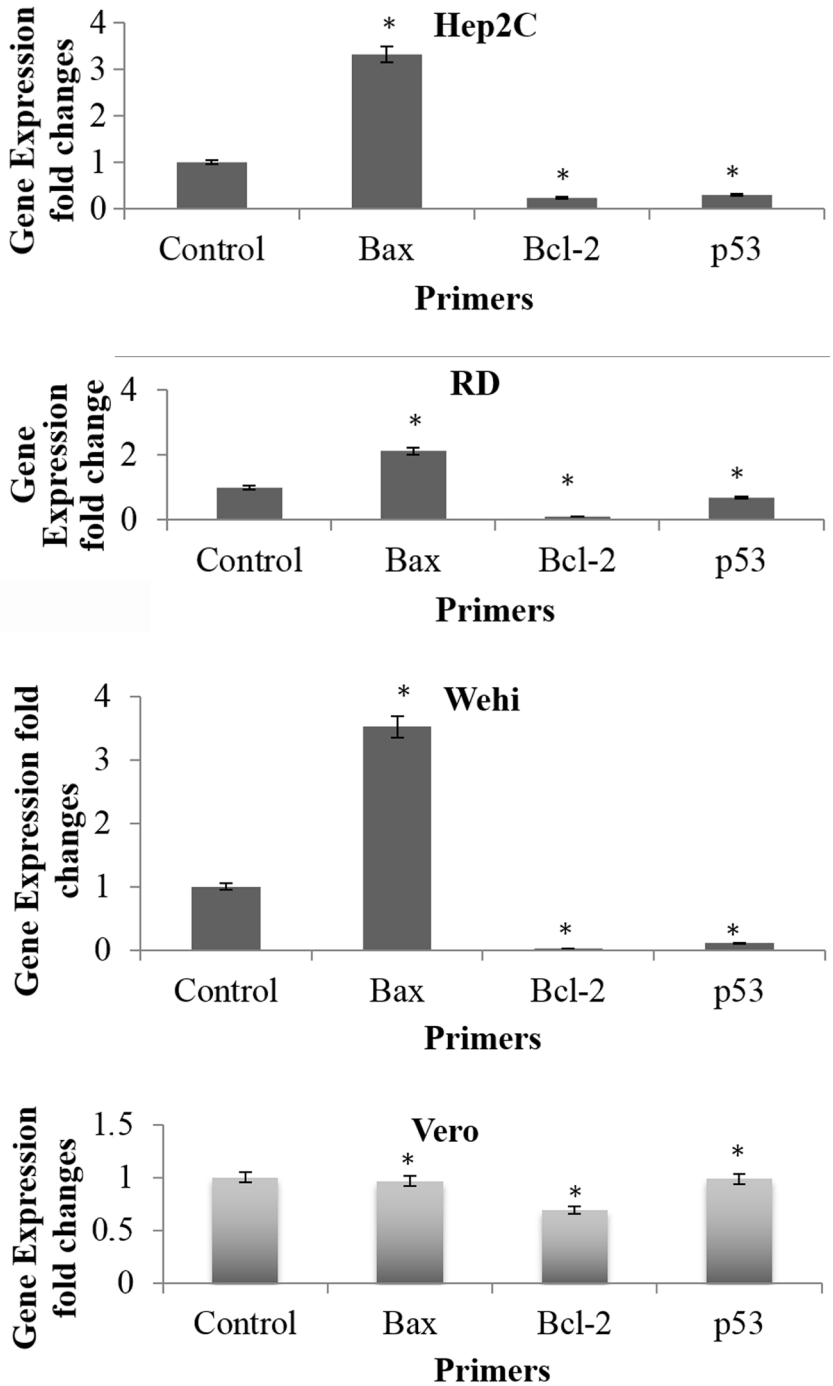

Figure 5. Usnic acid effect on mRNA levels of RD, Hep2C, HepG2, Wehi, and Vero cell lines. Expression level of usnic acid on mRNA was determined using qRT-PCR repeated three times after optimization. Values are statistically significant at *P < 0.05 compared to the respective control.

After the application of usnic acid, an approximately threefold decrease was seen in *Bax*; however, the *Bcl-2* antiapoptotic gene and *p53* tumor suppressor gene were decreased fivefold in the Hep2C cell line when compared to the control group ($P < 0.05$) (Figure 5a). There was an 8-fold and 2.5-fold decrease in the expression of the *Bcl-2* and *p53* genes in the RD cell line when compared to the control group, respectively ($P < 0.05$) (Figure 5b).

Usnic acid increased the transcripts of the *Bax* gene by twofold. Exposed to usnic acid, all examined suppressor gene levels were significantly underexpressed in the Wehi cell line. While there was an approximately 9.5- and 8.5-fold reduction in the gene expression of *Bcl-2* and *p53*, respectively, there was a 3.5-fold increase in *Bax* gene expression when compared to the control group ($P < 0.05$) (Figure 5c). It is obvious that all concentrations of usnic acid led to an increase in the *Bax* gene and decrease in *Bcl-2* and *p53* genes in mRNA levels when compared to the unexposed control sample. Contrary to cancer cells, there was not a significant decrease in gene expression in the Vero nonmalignant cell line ($P < 0.05$) (Figure 5d).

4. Discussion

It was shown in this study that there was no significant difference in the source of the usnic acid (whether natural or commercial) secondary metabolite against cancer lines. The results found in this study revealed that 6.25 and 25 µM usnic acid concentrations have important cytotoxic and apoptotic effects on cancer cell lines, whereas nonmalignant cell lines, particularly L929, maintain their life in spite of high doses of usnic acid. Almost a twofold increase occurred in *p53* and *Bax* expression for usnic acid, which suggests that usnic acid has tumor-inhibitory properties. This study confirmed that usnic acid plays a major role in the regulation of apoptosis of cancer cells. Therefore, our results add novel insight into the anticancer activity of lichen seconder metabolites.

Einarsdottir et al. investigated the inhibitory effect of pure (+)-usnic acid extracted from *Cladonia arbuscula* and (-)-usnic acid from isolated *Alectoria ochroleuca* on the proliferation of T-47D and Capan-2. It is of interest to compare the effects of the two enantiomers since different isomers of usnic acid can have different proliferation effects. No difference was determined between the two enantiomers of usnic acid (Einarsdottir et al., 2010). The present study compared natural usnic acid extracted from *Usnea diffracta* collected from Turkey with commercial usnic acid. Similar results were obtained for the two usnic acid types ($P < 0.05$). In this regard, it is noted that an effective novel drug source of usnic acid collected from *Usnea diffracta* lichen species will be possible for commercial production.

Chen et al. reported the role of autophagy on the toxicity of usnic acid. HepG2 cells were treated with different usnic acid concentrations and times (2, 6, 24, and 48 h) in order to determine the cytotoxic effect of usnic acid. It was found that usnic acid exhibits a time- and concentration-dependent growth-inhibiting effect, and lactate dehydrogenase (LDH) release occurred only after 24 h of exposure to 12.5 µM or 48 h of exposure to 3.13 µM usnic acid. No significant LDH release was observed after 2 or 6 h of exposure to the tested concentrations of usnic acid (Chen et al., 2014). In the present study, according to the MTT assay results, usnic acid exhibited a time- and concentration-dependent growth-inhibiting effect on the CaCo2, HepG2, Hep2C, RD, and Wehi cancer cell lines. In our study, it was observed for the HepG2 cell line that the most growth-inhibiting effect of usnic acid occurred after 48 h of exposure to 15 µM.

Singh et al. determined the anticancer potential of usnic acid in A549 cells. Usnic acid treatment (25, 50, and 100 µM) for 24 and 48 h resulted in a dose- and time-dependent decrease in cell growth and proliferation. In addition, the cell death effect was further increased after 48 h of treatment with usnic acid. As a result of our study, it was determined that 48 h of usnic acid treatment was the most active of all time parameters. Consistent with cell death data, it was found that usnic acid treatment for 48 h showed a dose-dependent increase in apoptotic cells (Singh et al., 2013). In the present study, usnic acid exposure was conducted at different concentrations and for 24, 48, and 72 h on various cancer cell lines. According to the MTT assay results, commercial and natural usnic acid secondary metabolites displayed cytotoxic effects on tested cell lines depending on the duration of exposure. Particularly after 48 and 72 h, 12.5 µM and higher concentrations of usnic acid significantly reduced cell viability ($P < 0.05$). The percentage of viable cells showed higher cytotoxic effects on cancer cell lines after treatment for 48 h with 25 µM commercial usnic acid or more.

Brisdelli et al. emphasized the effects of diffractaic acid, lobaric acid, usnic acid, vicanicin, variolaric acid, and protolichesterinic acid lichen metabolites on proliferation and viability of MCF-7, HeLa, and HCT-116. Cell viability reducing activity of usnic acid on HeLa and HCT-116 cells was found to be in the concentration range of 25–100 µM. It has also been found that usnic acid is the most potent cytotoxic agent on MCF-7, HeLa, and HCT-116 cells (Brisdelli et al., 2013). In our study, the most cytotoxic effect of treatment with commercial and natural usnic acid was observed on RD and Wehi cells. Usnic acid could potentially represent an alternative source for cancer therapy.

Backorova et al. extensively studied the antiproliferative/cytotoxic effects of parietin, atranorin, usnic acid, and gyrophoric acid lichen secondary metabolites in A2780, HeLa, MCF-7, SK-BR-3, HT-29, HCT-116 p53 +/+, HCT-116 p53, HL-60, and Jurkat cancer cell lines. They found that usnic acid was the most effective secondary metabolite with an approximately 50 µM usnic acid concentration effect on all cell lines (excluding HeLa cells and HCT-116 p53 +/+). Usnic acid and atranorin were demonstrated to induce apoptosis and inhibit cell proliferation in all tested cell lines (Backorova et al., 2011). In our study, it

was found that usnic acid after 48 h of exposure to 6–25 µM concentration showed a potent cytotoxic effect on all tested cell lines.

Koparal et al. investigated the cytotoxic activities of (+)-usnic acid and (-)-usnic acid on V79, A549, and human lymphocyte cells. They found that 100 µg mL^{-1} (+)-usnic acid and 100 and 50 µg mL^{-1} (-)-usnic acid concentrations induced cytotoxic effects in human lymphocytes. A significant increase in apoptotic cells was found at 25 and 30 µg mL^{-1} (-)-usnic acid and 50 µg mL^{-1} (+)-usnic acid concentrations. According to MTT assay results, (+)-usnic acid showed relatively more cytotoxic effects than (-)-usnic acid in A549 cells. Even at low doses of usnic acid, the two enantiomers were found to be more effective in the death and control of cancer cells compared to nonmalignant cell lines (Koparal et al., 2006). In our study, treatment with usnic acid showed a less cytotoxic effect on L929 and Vero cells than the cancer cell lines. The effect of usnic acid was confirmed to beat very low rates in nonmalignant cell lines by both MTT assay and qRT-PCR at the mRNA level ($P < 0.05$).

Apoptosis could occur as a result of many mechanisms. Agents suppress the proliferation of cancer cells via enhancing apoptosis. Afterwards, cancer prevention and treatment is ensured through these agents. To the best of our knowledge, this is the first study on the apoptosis-related gene expression properties of usnic acid against different cancer and nonmalignant cell lines.

Backorova et al.explored the apoptotic effects of parietin, atranorin, usnic acid, and gyrophoric acid on A2780 and HT-29 cancer cell lines. It was found that usnic acid and atranorin are more effective anticancer metabolites when compared with parietin and gyrophoric acid. The 50 and 100 µM concentrations of usnic acid remarkably increased the proportion of annexin V-positive cells after 24 h of exposure or more. It has been shown that usnic acid and atranorin are activators of programmed cell death in A2780 and HT-29 cells as a result of the detection of PARP, p53, Bcl-2/Bcl-xL, Bax, p38, and pp38 protein expressions (Backorova et al., 2012). Furthermore, we also demonstrated gene expression of usnic acid in cancer and nonmalignant cell lines without protein data. In the past decades, many studies focused on cytotoxic effects of usnic acid. However, our study is scientifically validated by mRNA levels for apoptosis mechanisms. This study determined that usnic acid inhibits cell growth in several types of cancer cells by regulating genes related to apoptosis. Similar observations were made by Zu et al. by qRT-PCR for emodin (Zu et al., 2015). They found that emodin is significantly increased with *Bax* levels and decreased with *p53* and *Bcl-2* ($P < 0.05$). The study by Zu et al. and our study provide similar results of alternative source-induced apoptosis of cancer cells.

Song et al. found that treatment with usnic acid significantly suppresses mouse corneal neovascularization via inhibiting vascular endothelial growth factor (VEGF)-activated VEGFR2 phosphorylation (Song et al., 2012). It was determined that usnic acid functions as a tumor angiogenesis inhibitor by suppressing VEGFR2-mediated AKT and extracellular signal-regulated kinase (ERK 1/2) signaling pathways. They also established that usnic acid does not only remarkably inhibit endothelial cell proliferation, migration, and tube formation, but also induces morphological changes and apoptosis in endothelial cells (Song et al., 2012). Usnic acid showed apoptotic effects on cancer cell lines tested in our study with the *Bax*, *Bcl-2*, and *p53* genes. After treatment with usnic acid, the most reduction of expressions of *Bcl-2* and *p53* genes was in the Wehi cell line with 9.5-fold decrease in *Bcl-2* when compared to the control group ($P < 0.05$). Thus, the most apoptotic effect of usnic acid occurred in this cell line. The most commonly mutated gene was *p53*, which regulates the cell cycle and has a significant role in the recovery of damaged cells by apoptosis. According to the results of this study, the expression levels of *p53* significantly decreased exposure to usnic acid at the EC_{50} level ($P < 0.05$). Therefore, our study revealed that usnic acid induced apoptosis by regulating proapoptotic genes.

Wang et al. analyzed five most-used reference genes and ten anticancer drug-related genes in tissue samples from lung, rectal, colon, gastric, esophageal, and breast tumors using qRT-PCR. They found that most tested target genes, except for *HER2*, display high expression levels in lung, rectal, and colon tumors and low expression levels in breast, gastric, and esophageal tumors. It was determined that *HER2* shows the highest expression level in breast tumors and the lowest expression in esophageal tumors. Moreover, lung cancer had the highest epidermal growth factor receptor (*EGFR*) expression level among the tumors while breast cancer had the lowest *EGFR* expression level (Wang et al., 2015). In the present study, *Bax*, *Bcl-2*, and *p53* gene expression levels in cancer cell lines (Hep2C, RD, and Wehi) and a nonmalignant cell line (Vero) were researched. According to the results, *Bax*, *Bcl-2*, and *p53* gene expression in the Vero cell line reached the highest levels for nonmalignant cell lines when compared with controls ($P < 0.05$). The changes in *p53* gene expression in the Wehi cell line was found to have the highest levels in all examined cancer cell lines. All tested gene expression levels were found to be low in the Hep2C cell line when compared with other examined cancer cell lines (RD and Wehi) ($P < 0.05$).

In conclusion, our study showed that usnic acid inhibited different cancer growths in vitro. In order to understand the molecular mechanisms of the apoptosis pathway, usnic acid secondary metabolites were studied by qRT-PCR. To

date, no study has investigated the gene expression profiles of cancer cells treated with usnic acid. As a result of our study, the antiapoptotic gene *Bcl-2* was downregulated and the proapoptotic gene *Bax* was upregulated after the examined cancer cells were treated with usnic acid. Studies have suggested that usnic acid might play a significant role in the apoptosis pathway. This study revealed that usnic acid seems to be suitable as an anticancer therapy to solve this public health problem. With further studies on additional molecular mechanisms and further clinical testing to pinpoint the anticancer activity of lichen species, their secondary metabolites can be revealed, and thus usnic acid could be used in cancer chemotherapy.

Acknowledgment
We would like to thank the Ankara University Project Offices, Project No. 16H0415002, for the partial financial support.

References

Alhazmi MI, Hasan TN, Shafi G, Al-Assaf AH, Alfawaz MA, Alshatwi AA (2014). Roles of p53 and caspases in induction of apoptosis in MCF-7 breast cancer cells treated with a methanolic extract of *Nigella sativa* seeds. Asian Pac J Cancer P 15: 9655-9660.

Ari F, Aztopal N, Oran S, Bozdemir S, Celikler S, Ozturk S, Ulukaya E (2014). *Parmelia sulcata* Taylor and *Usnea filipendula* Stirt induce apoptosis-like cell death and DNA damage in cancer cells. Cell Proliferat 47: 457-464.

Backorova M, Backor M, Mikes J, Jendzelovsky R, Fedorocko P (2011). Variable responses of different human cancer cells to the lichen compounds parietin, atranorin, usnic acid and gyrophoric acid. Toxicol In Vitro 25: 37-44.

Backorova M, Jendzelovsky R, Kello M (2012). Lichen secondary metabolites are responsible for induction of apoptosis in HT-29 and A2780 human cancer cell lines. Toxicol In Vitro 26: 462-468.

Behera BC, Adawadkar B, Makhija U (2006). Tissue-culture of selected species of the Graphis lichen and their biological activities. Fitoterapia 77: 208-215.

Brisdelli F, Perilli M, Sellitri D, Piovano M (2013). Cytotoxic activity and antioxidant capacity of purified lichen metabolites: an in vitro study. Phytother Res 27: 431-437.

Cansaran D, Atakol O, Halıcı MG, Aksoy A (2007). HPLC analysis of the usnic acid in some *Ramalina* species from Anatolia and ınvestigation of their antimicrobial activities. Pharm Biol 45: 77-81.

Chen S, Dobrovolsky VN, Liu F (2014). The role of autophagy in usnic acid-induced toxicity in hepatic cells. Toxicol Sci 142: 33-44.

Coskun ZM, Ersoz M, Acikgoz B, Karalti I, Cobanoglu G, Sesal C (2015). Anti-proliferative and apoptotic effects of methanolic extracts from different *Cladonia* species on human breast cancer cells. Folia Biol-Prague 61: 97-103.

Einarsdottir E, Groeneweg J, Björnsdottir GG (2010). Cellular mechanisms of the anticancer effects of the lichen compound usnic acid. Planta Med 76: 969-974.

Florea AM, Busselberg D (2011). Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. Cancer 3: 1351-1371.

Hanahan D, Weinberg RA (2011). Hallmarks of cancer, the next generation. Cell 144: 646-674.

Kılıç N, Değerli E, Torun V, Altaytaş F, Cansaran-Duman D (2016). Investigation of synergistic effect of tamoxifen and usnic acid on breast cancer cell line. JSM Biology 1: 1006.

Kim H, Kim KK, Hur JS, Upreti DK (2015). Anticancer activity of lichen metabolites and their mechanisms at the molecular level. In: Upreti DK, Divakar PK, Shukla V, Bajpai R, editors. Recent Advances in Lichenology. Berlin, Germany: Springer, pp. 201-208.

Koparal AT, Ayaz-Tüylü B, Türk H (2006). In vitro cytotoxic activities of (+)-usnic acid and (−)-usnic acid on V79, A549, and human lymphocyte cells and their non-genotoxicity on human lymphocytes. Nat Prod Res 20: 1300-1307.

Motomura M, Kwon KM, Suh SJ (2008). Propolis induces cell cycle arrest and apoptosis in human leukemic U937 cells through Bcl-2/Bax regulation. Environ Toxicol Pharmacol 26: 61-67.

Newman DJ (2008). Natural products as leads to potential drugs: an old process or the new hope for drug discovery? J Med Chem 51: 2589-2599.

Nguyen TT, Somy Yoon, Yang Y (2014). Lichen secondary metabolites in *Flavocetraria cucullata* exhibit anti-cancer effects on human cancer cells through the ınduction of apoptosis and suppression of tumorigenic potentials. PLoS One 9: e111575.

Purvis WO, Coppins BJ, Hawksworth DL, James PW, Moore DM (1992). The Lichen Flora of Great Britain and Ireland. London, UK: Natural History Museum Publication.

Saleem M, Nazir M, Ali MS, Hussain H, Lee YS, Riaz N, Jabbar A (2010). Antimicrobial natural products: an update on future antibiotic drug candidates. Nat Prod Rep 27: 238-254.

Singh N, Nambiar D, Kale RK, Singh RP (2013). Usnic acid inhibits growth and induces cell cycle arrest and apoptosis in human lung carcinoma A549 cells. Nutr Cancer 65: 36-43.

Song Y, Dai F, Zhai D, Dong Y (2012). Usnic acid inhibits breast tumor angiogenesis and growth by suppressing VEGFR2-mediated AKT and ERK1/2 signaling pathways. Angiogenesis 15: 421-432.

Stanojkovic T (2015). Investigations of lichen secondary metabolites with potential anticancer activity. In: Rankovic B, editor. Lichen Secondary Metabolites. Berlin, Germany: Springer, pp. 127-41.

Stocker-Wörgötter E (2001). Experimental lichenology and microbiology of lichens: culture experiments, secondary chemistry of cultured mycobionts, resynthesis, and thallus morphogenesis. Bryologist 104: 576-581.

Stocker-Wörgötter E, Elix JA (2002). Secondary chemistry of cultured mycobionts: formation of a complete chemosyndrome by the lichen fungus of *Lobaria spathulata*. Lichenologist 34: 351-359.

Wang H, Yang B, Geng T, Li B, Dai P, Chen C (2015). Tissue-specific selection of optimal reference genes for expression analysis of anti-cancer drug-related genes in tumor samples using quantitative real-time RT-PCR. Exp Mol Pathol 98: 375-381.

Wirth V (1995). Die Flechten Baden-Württembergs. Teil 12. Berlin, Germany: Eugen Ulmer GmbH (in German).

Yamamoto Y, Miura Y, Higuchi M, Kinoshita Y, Yoshimura I (1993). Using lichen tissue cultures in modern biology. Bryologist 96: 384-393.

Yamamoto Y, Mizuguchi R, Takayama S, Yamada Y (1987). Effects of culture conditions on the growth of usneaceae lichen tissue cultures. Plant Cell Physiol 28: 1421-1426.

Yamamoto Y, Mizuguchi R, Yamada Y (1985). Tissue cultures of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. Agr Biol Chem Tokyo 49: 3347-3348.

Zu C, Zhang M, Xue H, Xiaopeng C (2015). Emodin induces apoptosis of human breast cancer cells by modulating the expression of apoptosis-related genes. Oncology Letters 10: 2919-2924.

Zuo S, Wang L, Zhang Y, Zhao D, Li Q, Shao D, Fang X (2015). Usnic acid induces apoptosis via an ROS-dependent mitochondrial pathway in human breast cancer cells in vitro and in vivo. RSC Adv 5: 153-162.