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Research Article

Usnic Acid Reduces Colon Cancer Cell Viability and Colony Formation by Affecting Cancer Cell Metabolism

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Abstract: Today, some natural and plant-based products are of great interest, at least as supplements, in cancer prevention and treatment due to their anti-cancer properties. One of them is usnic acid, which is a secondary metabolite synthesized by the polyketide pathway in Usnea lichen species and is a promising agent for cancer treatment. In this study, the effects of usnic acid on cell proliferation, colony formation, and cancer metabolism-related Sirtuin 2 (SIRT2) and lactate dehydrogenase (LDH) enzymes in COLO-205, a colon cancer cell line, were investigated. COLO-205 cells were exposed to three different doses of usnic acid: 15 µM, 30 µM, and 60 µM for 24 h. Usnic acid treatment reduced colon cancer cell viability at a dose dependent manner. The highest dose of usnic acid treatment (60 µM) decreased cell viability by about 40%. It has been determined that a 15 µM dose of usnic acid reduces cell viability by about half, and a dose as low as 1 µM reduces the colony-forming abilities of cancer cells by about half. It has been determined that usnic acid reduces the anti-cancer effect seen in this cell line, at least in part, by altering SIRT2 and LDH protein expressions, thus affecting cancer metabolism.

Keywords: *Cancer, Cell culture, Colon, LDH, SIRT2, Sirtuin*

Usnik Asit Kanser Hücre Metabolizmasını Etkileyerek Kolon Kanseri Hücre Canlılığını ve Koloni Oluşumunu Azaltır

Öz: Günümüzde bazı doğal ürünler, anti-kanser özelliklerinin bulunmasının nedeniyle, kanser önleme ve tedavisinde, en azından takviye olarak büyük ilgi görmektedir. Bunlardan birisi de Usnea liken türünde poliketid yolu ile sentezlenen bir ikincil metabolit olan usnik asittir ve kanser tedavisi için umut verici bir ajandır. Bu çalışmada, usnik asidin bir kolon kanseri hücre hattı olan COLO-205'te hücre proliferasyonu, koloni formasyonu ve kanser metabolizmasıyla ilişkili Sirtuin 2 (SIRT2) ve laktat dehidrojenaz (LDH) enzimleri üzerine etkileri araştırılmıştır. COLO-205 hücreleri, 24 saat boyunca üç farklı usnik asit dozuna maruz bırakıldı: 15 µM, 30 µM ve 60 µM. Usnik asit tedavisi, doza bağlı bir şekilde kolon kanseri hücresi canlılığını azalttı. En yüksek doz usnik asit muamelesi (60 µM), hücre canlılığını yaklaşık %40 oranında azalttı. Usnik asidin 15 µM dozunun hücre canlılığını yaklaşık yarı yarıya azalttığını ve 1 µM kadar düşük dozlarda ise kanser hücrelerinin koloni oluşturma yeteneklerini yaklaşık yarı yarıya düşürdüğü belirlenmiştir. Usnik asidin bu hücre hattında görülen anti-kanser etkisi, en azından kısmen, SIRT2 ve LDH protein ifadelerini değiştirerek, dolayısıyla kanser metabolizmasına etki ederek azalttığı belirlenmiştir.

Anahtar sözcükler: *Hücre kültürü, Kanser, Kolon, LDH, SIRT2, Sirtuin*

Introduction

Cancer ranks second place on the list of deadliest diseases in the world; however, knowledge about the molecular mechanism of cancer is still lacking. Statistical studies show that the disease is increasing exponentially and is

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becoming significantly dangerous [1,2]. Colorectal cancer is one of the most common types of all cancer kinds [2]. Modern treatment methods, such as chemotherapy, radiation therapy, and immunotherapy are widely applied to treat many types of cancer; however, the importance of herbal extracts and phytochemicals complementing to conventional treatments is increasing in the fight against cancer [3,4]. One of the reasons that lead researchers to seek alternative treatments is the high side effects and resistance of some cancer cells to chemotherapy. In the light of recent studies, it has been claimed that therapeutic metabolites obtained from organisms existing in nature are an unlimited source for future drug production [5]. One of the alternative treatment methods to treat cancer could be applications with usnic acid obtained from lichens.

Complex organisms formed by the combination of fungi, algae, and cyanobacteria with a symbiotic bond are called lichens [6]. These organisms, which are known to have more than 17.000 species and are resistant to harsh living conditions, live on the soil surface or in the bark of trees. The most famous lichen secondary metabolite is usnic acid. Usnic acid can be easily isolated in high product purity and has various properties, such as anti-inflammatory, analgesic, wound healing, antioxidant, anti-microbial, anti-protozoal, and anti-viral [7,8]. Lichens synthesize hundreds of metabolites and these metabolites have been widely used due to their therapeutic effects [9]. Its promising effects on cancer treatment were first identified in studies on mice for the treatment of Lewis lung cancer [10]. In addition, cytotoxic and mutagenic activities of usnic acid were detected on normal and malignant human cell lines [11]. However, it is poorly known the molecular mechanisms of how usnic acid shows its therapeutic effect on cancer cells.

Studies have shown that the sirtuin (SIRT) protein family has important effects on the occurrence of several age-associated diseases, including cancer [12,13]. SIRTs regulate a variety of cellular functions, including metabolism, aging, inflammation, differentiation, stress response, and mitochondrial biogenesis [14,15]. SIRT2 is mainly found in the cytoplasm and plays important roles in the cell cycle and regulation of metabolism [16,17]. SIRT2 deacetylates and increases lactate dehydrogenase (LDH). LDH is an important enzyme for energy metabolism and catalyzes the conversion of pyruvate to lactate in the anaerobic metabolic pathway. LDH might be up-regulated in serum and tissues of patients diagnosed with cancer and is believed to associate with higher cancer cell proliferation rate and survival [18].

This study aimed to observe the effects of usnic acid on SIRT2 and LDH in colon cancer cell lines to understand the mechanism of action based on its known positive effects on cancer.

Material and Methods

Cell Cultures

COLO-205 colon cancer cell lines were maintained in RPMI-1640 (Roswell Park Memorial Institute-1640) (Gibco, USA) growth medium which contains 10% fetal bovine serum (FBS) and 1% antibiotic at 37°C, in a sterile incubator including 5% CO_2.

Cell Viability Assays

Cancer cells ($1x10^3$) were seeded in sterile 96-well microplates which contain a 100 µL growth medium in each well. After 24 h, cells were treated with 15 µM, 30 µM, and 60 µM doses of usnic acid (Santa Cruz Biotechnology, U.S.A.) for 24 h. The doses were selected according to a previous report [19]. To determine the cell viability, the 10 µL cell viability test kit (ECOTECH, CVDK-8, Turkey) was added to each well-containing 100 µL medium, and absorbance was measured at 450 nm with a spectrophotometer. In addition, the cells were photographed via an inverted microscope (Invitrogen, USA).

Homogenization of Usnic Acid-Treated Colon Cancer Cells

At the end of treatments, cells were collected and stored at -80°C until the homogenization procedure. A lysis buffer containing 1:1000 phenylmethylsulphonyl fluoride (PMSF) proteinase inhibitor was added to each cell sample. Finally, samples were centrifuged at 1000 rpm for 5 min and the supernatant portion was taken, and the total protein concentrations of the samples were determined by the Bradford assay. This assay is a spectroscopic procedure to measure total protein concentration in our homogenates. The assay solution includes Coomassie Brilliant Blue G-250 dye, and upon binding to proteins, the color of the dye and its absorption changes from 465 to 595 nm in response to various concentrations of protein. BSA standards were prepared using 1:1 serial dilutions from 2 to 0.0625 mg/mL.

Determination of Protein Expression with Western Blotting

TGX Stain-Free Fast Acrylamide SDS gel (10%) is prepared following the manufacturer's protocols (BIO-RAD, USA). Forty micrograms of each sample including 4x loading dye were heated at 95°C for 5 min. These samples were loaded into the wells and run at 120V for 120 min. Afterward, the transfer of membranes from the gel to a PVDF membrane was performed at 150 mA. Following the transfer procedure, the blotted PVDF membrane was blocked with 5% dry milk for 30 min, and then incubated with 1:1000 diluted SIRT2 (Sigma, USA), and LDH (Cell Signaling) primary antibodies for 16 h at 4°C. After the overnight incubation period,

the membrane was washed with three times phosphate-buffered saline including 0.1% Tween 20 (PBS-T). The secondary antibody incubation was performed at a 1:10000 dilution (Abcam, USA) at room temperature for 30 min. After secondary antibody treatment, membranes were washed again three times and were visualized with the Immobilon ECL Ultra Western HRP Substrate kit (Merck, USA).

Colony Formation Assay

To examine the effects of usnic acid doses on colony formation in COLO-205 colon cancer cells, cells were seeded on sterile 6-well plates with a 2 mL growth medium with 200 cells per well (n=6) as described previously [20]. At the end of 72 h, the growth medium in each well was withdrawn and replaced with a fresh growth medium containing usnic acid at doses of 1 µM, 5 µM, and 15 µM. The experiment was continued for 14 days in total by replacing the media in the wells with fresh medium containing usnic acid at the indicated doses at three-day intervals. The reason why the amounts of this dose determined in colony formation are different from the dose amounts used in the study is due to the fact that no cell colony was observed in the 15 µM dose application determined in the study. In our colony formation preliminary study, it was observed that these doses destroyed the colony formation, and therefore it was deemed appropriate to use low doses. At the end of the experiment, the medium in each well was discarded to determine the colony formations, and each well was washed with PBS and incubated with 1 mL of the fixative solution (1 acetic acid: 7 methanol) for 2 min in room temperature. At the end of the period, the fixative solution was removed from the wells and the samples were stained with crystal violet. At the end of the staining, the dye residues in the wells were removed and colony staining was obtained with the help of an imaging system. Transferring the colony formations to numerical data was performed with the Particle Tool in ImageJ 1.53m software.

Statistical Analysis

The data set was created by recording all the numerical data obtained with IBM SPSS 26.0 software. All the data in the data set were first evaluated according to the consistency of the parametric test assumptions and then the results of the homogeneity tests. In line with the obtained significance values, One-Way ANOVA was used to analyze the data. The probability value $P<0.05$ was accepted as a significance level in all statistical tests. Graphs were created by using the mean and standard deviation values.

Results

Usnic Acid Reduced the Cell Viability of Colon Cancer Cells

The effects of 15 µM, 30 µM, and 60 µM usnic acid doses applied to COLO-205 colon cancer cells for 24 h on cell survival are shown in *Fig. 1*. The data we obtained showed that the percentage of cell survival decreased in a dose-dependent manner of usnic acid, and all these reductions were significant at the $P<0.001$ level compared to the control and DMSO groups. Almost 50% of colon cancer cells died in response to as low as 15 µM usnic acid.

The images of COLO-205 colon cancer cells, which were exposed to usnic acid for the specified time, are shown in *Fig. 2*. The growth of COLO-205 significantly decreased especially at 60 µM usnic acid. Some cellular damage was detectible after 24 h of treatment of usnic acid in a dose-dependent manner. Non-treated, control cells had a round shape and their sizes were very similar. Treatment of usnic acid killed the cells and some debris were more common around the cells.

Protein Expressions of SIRT2 and LDH After Treatment with Usnic Acid

The effects of the indicated usnic acid doses on SIRT2 and LDH protein amounts in COLO-205 colon cancer cells

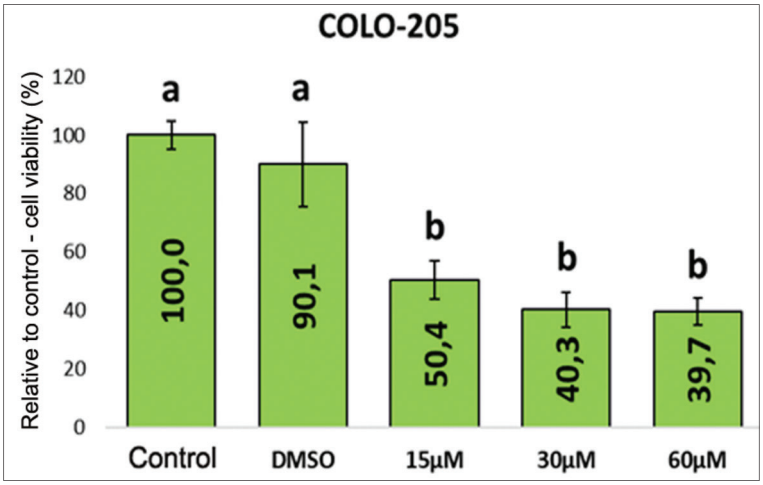

Fig 1. Effects of usnic acid on COLO-205 colon cancer cell survival after 24 h (One-Way ANOVA: $P<0.001$, posthoc Bonferroni: $P<0.001$). The experiment was repeated at least three times

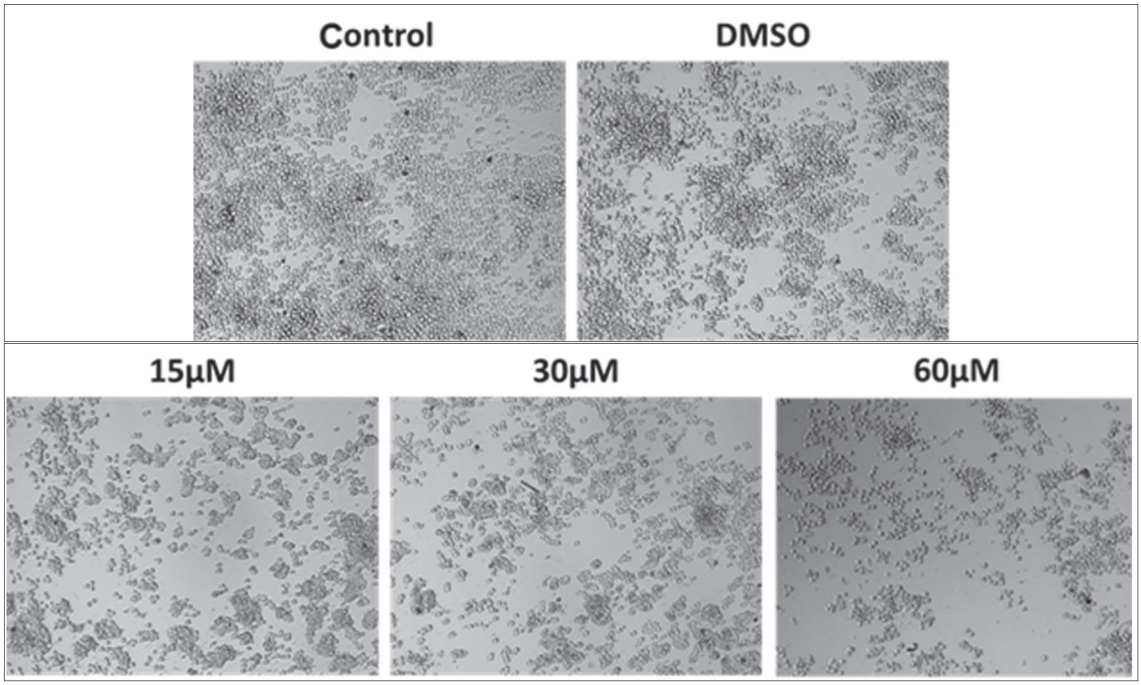

Fig 2. Inverted microscope images (40X magnification) were obtained at the end of the 24 h of COLO-205 colon cancer cells exposed to 15 µM, 30 µM, and 60 µM usnic acid. Representative images are shown

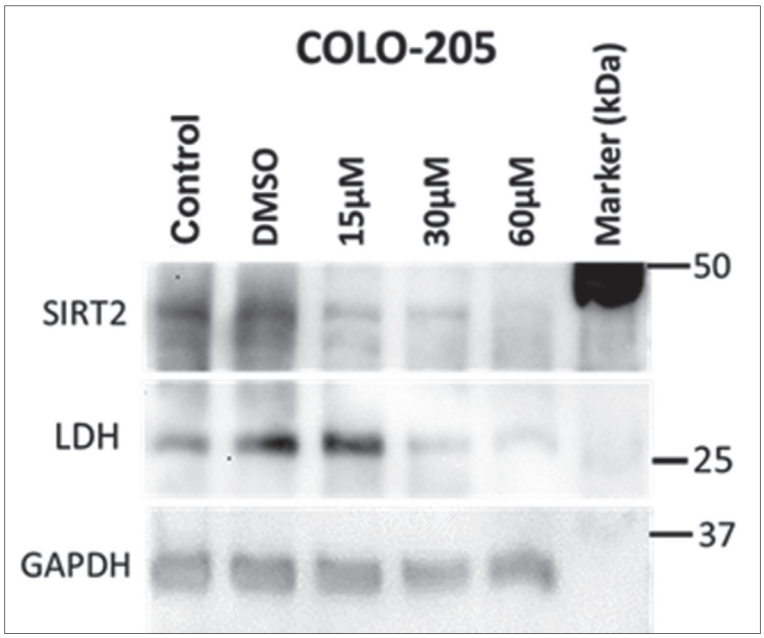

Fig 3. The effects of 15 µM, 30 µM, and 60 µM usnic acid doses were applied to COLO-205 colon cancer cells on SIRT2 and LDH protein amounts at the end of the 24 h. GAPDH was used as the loading control. The experiment was repeated at least three times

were investigated. The GAPDH was used as a loading control *(Fig. 3)*. The data obtained as a result of the Western blotting analysis showed that SIRT2 protein expression decreased with usnic acid application compared to the control group. In addition, it was observed that usnic acid decreased SIRT2 protein amounts inversely with increasing dose administration. It was observed that 60 µM usnic acid dose application dramatically reduced the amount of SIRT2 protein in COLO-205 cells. Similarly, the amount of LDH protein in COLO-205 colon cancer cells reduced drastically in response to both 30 and 60 mM usnic acid.

The Effect of Usnic Acid on Colony Formations of Colon Cancer Cells

With this experiment, it was aimed to measure the anti-proliferative and transformation potential of usnic acid. The effects of 1 µM, 5 µM and 15 µM usnic acid doses applied to COLO-205 colon cancer cells on the colony formation numbers of the cells at the end of the 14 days

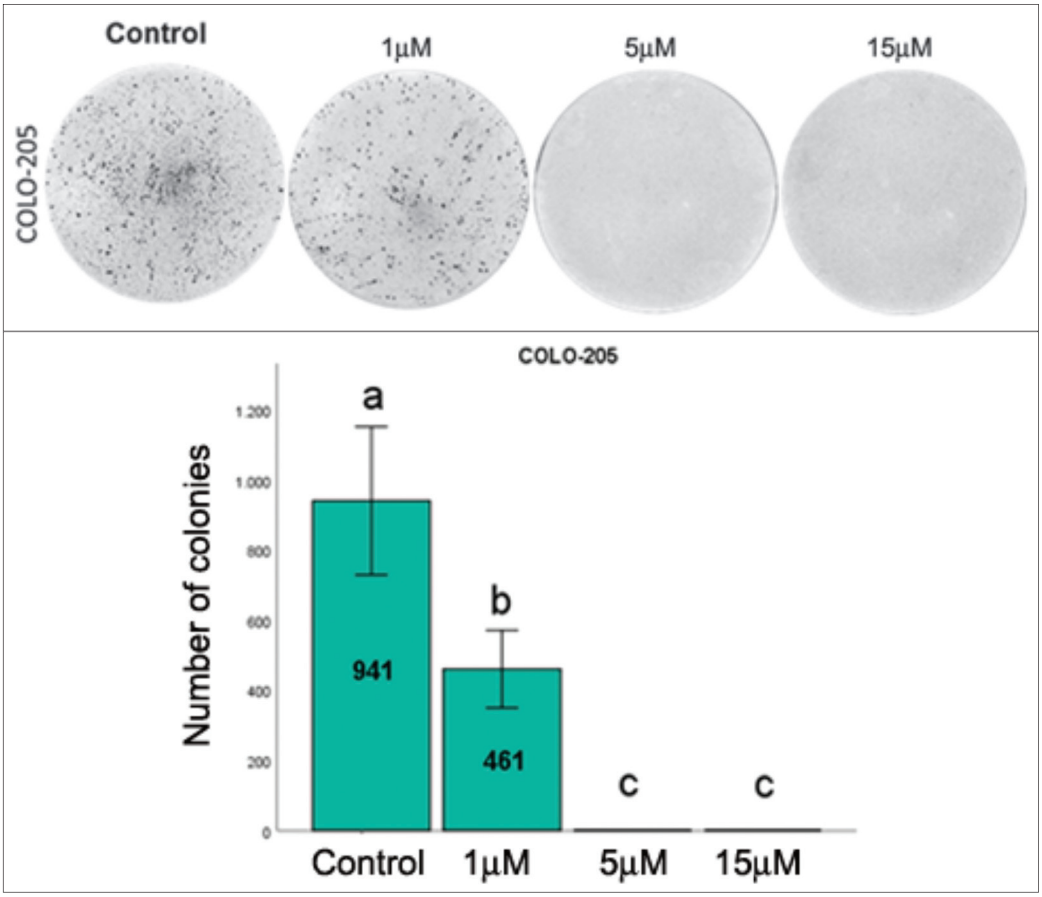

Fig 4. The effects of 1 µM, 5 µM, and 15 µM usnic acid application on COLO-205 cells on colony formation numbers at the end of the 14 days (One-Way ANOVA: P<0.001, PostHocTamhane's T2: pab, ac, bc<0.01)

are shown in *Fig. 4 (lower panel)*, and the images of the colony formations obtained at the end of the experiment are shown in *Fig. 4 (upper panel)*. The reason why the doses of usnic acid used in colony formation differ from the hypothetical experimental doses is that these doses (15 µM, 30 µM, and 60 µM) completely prevented the formation of colony formation in COLO-205 colon cancer cells in the preliminary study. For this reason, the dose densities used in our colony formation experiment were redesigned to include the lowest of the hypothetical dose densities, 15 µM. The data obtained as a result of the colony formation analysis show that there is statistical significance at the P<0.01 level between all administered dose groups and the control group *(Fig. 4)*. In addition, it was observed that COLO-205 colon cancer cells could not form any colony formation at 5 µM and 15 µM usnic acid doses *(Fig. 4)*. These results showed that an usnic acid concentration as low as 1 µM significantly reduces the ability of a single COLO-205 cell to develop into a colony.

Discussion

Previous studies have reported that usnic acid displays some anti-cancer activities by inducing apoptosis and decreasing apoptosis *in vitro* and *in vivo* [6,19]. The data obtained with this study showed that usnic acid lowers cell viability and colony formation ability of the colon cancer cells. In COLO-205 cell lines, usnic acid decreases the amount of both SIRT2 and LDH proteins in a dose-dependent manner.

The roles of SIRT2 in cancer are context-dependent, and both tumor suppressor and tumor-promoting activities of SIRT2 were reported [12,17,21]. Some studies have reported that low expression of SIRT2 as a deacetylase protein with anti-cancer properties is associated with poor prognosis in cancer and this protein is downregulated in many cancer types [22]. In addition, mice with the SIRT2 gene silenced spontaneously develop tumors of different organ origins [23]. On the contrary to these studies, some studies have reported that SIRT2 is up-regulated in various cancer types [24].

Cancerous cells gain many advantages by producing hypoxia signals, and in this respect, promoting aerobic glycolysis in energy production [25]. It has been proposed that SIRT2 protein modulates the Warburg effect, which has an important incident in cancer cell proliferation, by increasing the amount of intermediate products involved

in the energy production system [16]. In the data obtained, it was seen that at high concentrations of usnic acid, COLO-205 cells had a reduced expression of SIRT2 in colon cancer cells. SIRT2 has several non-histone substrates associated with diverse signaling pathways and cellular events. One of the substrates of SIRT2, which plays an important role in cancer, is LDH. LDH is one of the key enzymes for the Warburg effect since most tumors display increased aerobic glycolysis and lactate production [20,26]. High levels of LDH stimulates cancer cells to proliferate at a higher rate by increasing angiogenesis and epithelial to mesenchymal transition (EMT). It has been reported that SIRT2 deacetylates and increases the enzymatic activity of LDH; consequently, promoting cell proliferation [27]. In our study, we determined that LDH protein expression is reduced in response to as low as 30 μM usnic acid treatment. Reducing LDH protein levels by usnic acid treatment possibly decreased the rates of aerobic glycolysis and decreased the survival of cancer cells. According to our cell viability assay, usnic acid kills almost half of the colon cancer cells when 15 μM usnic acid is used. Moreover, as low as 1 μM usnic acid is sufficient to significantly decrease the number of colonies. This means that in addition to LDH, some other mechanisms, maybe some other SIRT2 substrates, might play a role to exert the anti-proliferative activity of usnic acid.

In conclusion, in the light of all these findings, it cannot be said that usnic acid achieves fully its anti-cancer properties through its effects on LDH expression, but whether it might change the activities of other SIRT2 substrates or other sirtuins should be investigated with detailed studies. In addition, the effects of usnic acid on the sirtuin-mediated anti-cancer pathway may be different in different cancer cells and different cancer types should also be taken into account.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

The authors report no conflicts of interest.

Author Contributions

Study conception and design: T.A., B.Y., O.O.; acquisition of data: B.Y., R.D., O.O., M.I., S.Y., B.M.; analysis and interpretation of data: T.A., B.Y., O.O., R.D., H.B. M.I., B.M.; drafting of manuscript: O.O., B.Y., T.A., S.Y., B.M.; critical revision: T.A., B.Y., R.D., M.I., S.Y., H.B., B.M., O.O. Authors give final approval of the version: T.A., B.Y., R.D., M.I., S.Y., H.B., B.M., O.O.

Ethical Approval

Not necessary.

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