Süleyman Demirel Üniversitesi Fen Edebiyat Fakültesi Fen Dergisi https://dergipark.org.tr/sdufeffd
Süleyman Demirel University Faculty of Arts and Sciences Journal of Science e-ISSN: 1306-7575
Research Article, 2023, 18(3): 195-202, DOI: 10.29233/sdufeffd.1250542

Effects of Usnic Acid on Cytotoxicity, Colony Formation and Migration in SK-UT-1 Human Uterine Leiomyosarcoma Cells

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(Received: 13.02.2023, Accepted: 24.07.2023, Published: 23.11.2023)

Abstract: Usnic acid (UA) is a common lichenic secondary metabolite with therapeutic potential. Anticancer, antimicrobial, and antioxidant effects have been demonstrated and UA-enriched extracts are often used to treat various diseases in traditional medicine. First, we performed a viability assay of UA in human uterine leiomyosarcoma (SK-UT-1) since no *in vitro* cytotoxicity data have been reported so far. For this purpose, the cytotoxicity of UA on SK-UT-1 and embryonic kidney (HEK293) cells was studied at 24 and 48 hours. The toxicity of UA was investigated by the MTT test. In addition, we assessed UA colony formation and migration properties against SK-UT-1 cells in 6-well plates. Our results showed a significant cytotoxic effect of UA at the 5.34 µM; UA was also effective against colony formation ability and wound healing assay. In conclusion, our study suggests that UA exerts anti-proliferative effects on SK-UT-1 cells by inducing cell death. Moreover, our results suggest that the potential anticancer activities of UA should be further studied by additional molecular biological approaches.

Keywords: Usnic acid, Uterine leiomyosarcoma, SK-UT-1, Cytotoxicity, Colony formation, Migration

1. Introduction

Uterine sarcomas are tumors with a very low incidence among all uterine cancers. They constitute ~3% of all uterine cancers [1]. Uterine leiomyosarcoma (LMS) is rare cancer that originates from the smooth muscle cells in the uterus. LMS is an aggressive tumor with a high risk of recurrence and death [1, 2]. Pharmacological agents are very important for the treatment of uterine LMSs, and a limited number of drugs such as doxorubicin, gemcitabine, eribulin and docetaxel are used in the treatment of this disease [3, 4]. Current researches focus on the development of new effective agents and novel therapeutic strategies for the treatment of this severe neoplasia [5].

Secondary metabolites are present in plants, fungi, lichens, and bacteria. They contain powerful pharmacological properties that have been used in treating various diseases including cancer, from the past to the present [6-8]. UA is a secondary metabolite, a dibenzofuran derivative, which is found very common in lichens, especially in the Usnea genus [9, 10]. UA is one of the first discovered lichen secondary metabolites and has strong pharmacological and biological activities such as antioxidant, anticancer, antimicrobial, antiviral, and anti-inflammatory [10-12]. In previous studies, the

anticancer activities of UA and its derivatives have been investigated on different cancer cells such as endometrium [12], lung [13], breast [14], colorectal [15], leukemia [16], gastric [17], prostate and melanoma [18]. In all these studies, it has been reported that UA exhibits anti-carcinogenic activity through molecular biological mechanisms including stimulation of apoptosis, modulation of oxidative DNA damage, induction cell cycle arrest, suppression of cell proliferation and regulation of expression of various genes and non-coding RNAs [12-18].

In addition to all these studies, there is no study in the literature about the effectiveness of UA in LMS. The aim of this study is to evaluate the dose and time-dependent cytotoxic effect of UA in the human uterine LMS cells SK-UT-1 (HTB114), and also to reveal its effect on colony formation and migration under *in vitro* cell culture conditions.

2. Material and Method

2.1. Cell culture

The SK-UT-1 and HEK293 (embryonic kidney) cells were obtained from American Type Culture Collection (ATCC). Cells cultured with Dulbecco's Modified Eagle's Medium (DMEM) or Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, Germany) supplemented with 1% penicillin/streptomycin mix (Capricorn) and 10% fetal bovine serum (FBS) (Capricorn, Germany) as described previously [19].

2.2. MTT assay

Cytotoxic effects of UA on SK-UT-1 and HEK293 cells were determined by MTT (dimethyl thiazolyl tetrazolium bromide) (Merck, USA) assay as described previously [20]. SK-UT-1 and HEK293 cells were grown in 96-well plates (100 µL/well, $2x10^3$ cells) and maintained at 37°C in a humidified condition in a 5% CO_2 incubator under suitable conditions. Following 24-hour incubation, cells were treated with various concentrations (0.75, 1.5, 5, 10, and 25 µM) of UA (Sigma-Aldrich, Germany) dissolved in chloroform (CarloErba, France) (not exceeding 0.5%) 24 and 48 h. After incubation periods, 10 µL MTT solution (5 mg/mL in 100 µL culture media, Merck) was added and maintained at 37ºC. After 3 h incubation, formazan dye was dissolved in 50 µL of dimethyl sulfoxide (DMSO,Carlo Erba, Italy) for at least 30 minutes. At the end, the optical density (OD) was measured in a spectrophotometer (Epoch, BioTek, USA) at 590 nm.

2.3. Colony formation assay

Effects of UA on colony formation status in LMS cells were performed by colony formation assay described previously [21]. Briefly, cells ($1x10^3$ cells per 6-well plate) were seeded and treated with the IC50 dose of UA for 48 h. After 48 h exposure, mediums were refreshed and maintained for at least one week. The fixation of the cells was executed by 100% methanol for 10 min at -20ºC and stained with crystal violet (0.1%) at RT for 15 min. Colonies photographed under an inverted microscope (Oxion Inverso, Euromex, Holland) and counted with ImageJ software 1.53e (USA).

2.4. *In-vitro* scratch assay

The migration efficacy of the UA was investigated in SK-UT-1 cells. Briefly, $3x10^4$ cells were seeded on 6-well culture plates (Jet Biofil, China) and incubated to grow until confluent. After incubation, cells were scraped by a 200 µL tip. The wells were cleaned with phosphate-buffered saline (PBS, Bioshop, Poland) to remove detached cells. The culture medium was refreshed by media containing 5.34 µM UA for 48 h and the wound

area in the treated cells was compared with the control (cells treated with chloroform at an equal volume). The wound size was imaged (0, 24, 48, and 72 h) with an inverted microscope at 10x magnification, and wound closure rate (%) was calculated with ImageJ software.

2.5. Statistical analysis

GraphPad Prism v.9 (San Diego, CA, USA) was used to evaluate the differences between two groups. The differences in the groups were evaluated using the Student's t-test or One-way ANOVA. $P < 0.05$ was considered as statistically significant.

3. Results and Discussion

The cytotoxicity of UA was evaluated against human uterine cancer line SK-UT-1 and the non-carcinogenic HEK293 cell line by the MTT assay. In the current study, five different concentrations of UA (0.75 to 25 µM) were attended for cytotoxicity investigations for 24 and 48 h (Figure 1). The results show a significant reduction in proliferation rate in the SK-UT-1 cells with increasing the dose concentration for 48 h. After 48 h of exposure, approximately 87% of SK-UT-1 cells were eradicated by 25µM UA treatment, decrement in the survival of 56% for HEK293 cells. The cytotoxicity of UA (expressed as IC50 values) was found to be 5.34 µM for SK-UT-1 and 21.09 µM for HEK293 cells at 48 h.

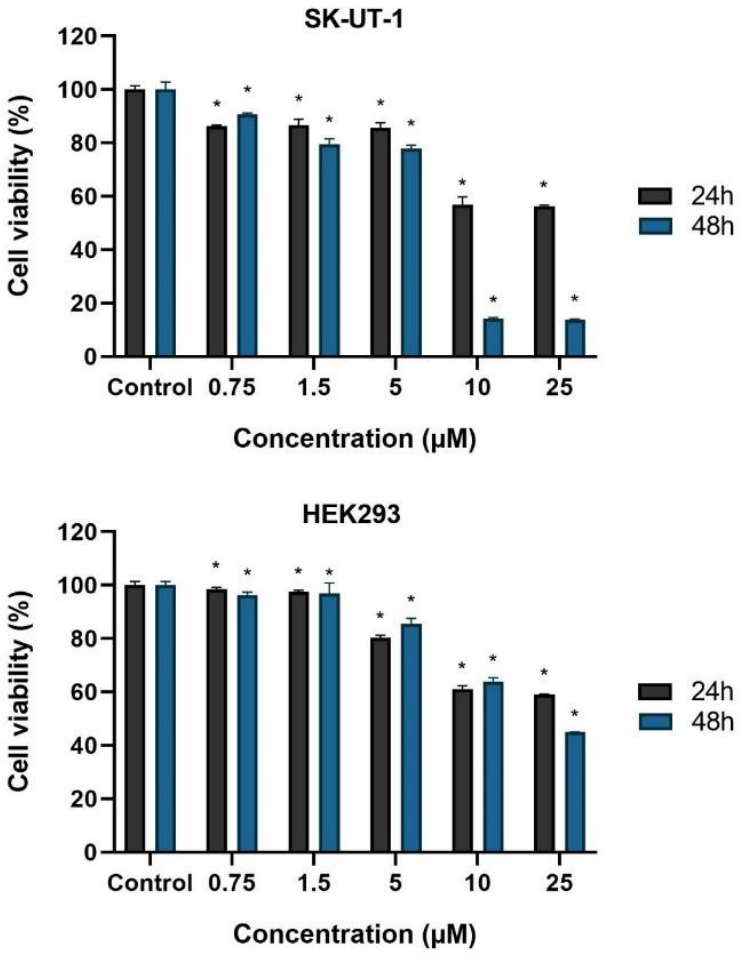

Figure 1. The cytotoxicity of UA was performed using the MTT assay of cancerous SK-UT-1 and embryonic kidney HEK293. Values are mean ± SD of triplicate value. *p <0.05 vs. control group.

The effects of UA on the proliferation of the SK-UT-1 cells were observed through colony formation assay. Figure 2 shows that the dose group treated with IC50 of UA was significantly reduced, suggesting that UA can effectively inhibit the proliferation of the uterine LMS cells.

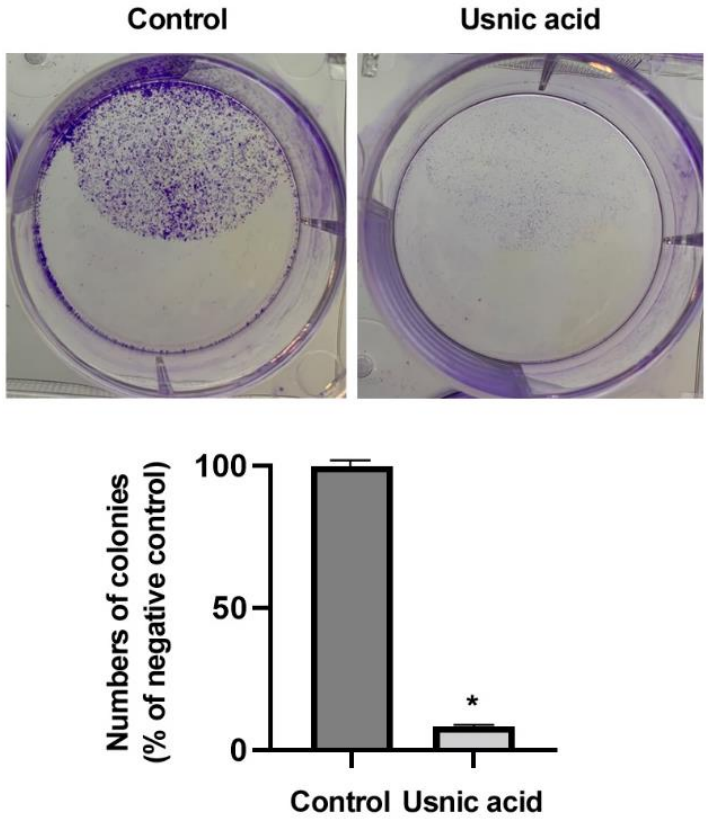

Figure 2. Colony formation assay. The number of colonies of the SK-UT-1 cells treated with UA vs. control group (containing chloroform v/v) for 48 h. *p < 0.05.

A scratch assay was carried out to assess the effect of UA on the migration of the SK-UT-1 cells. The migration of SK-UT-1 cells was significantly inhibited by UA and cell migration was restricted by 63% at the end of 72 h compared to control (Figure 3, p <0.05).

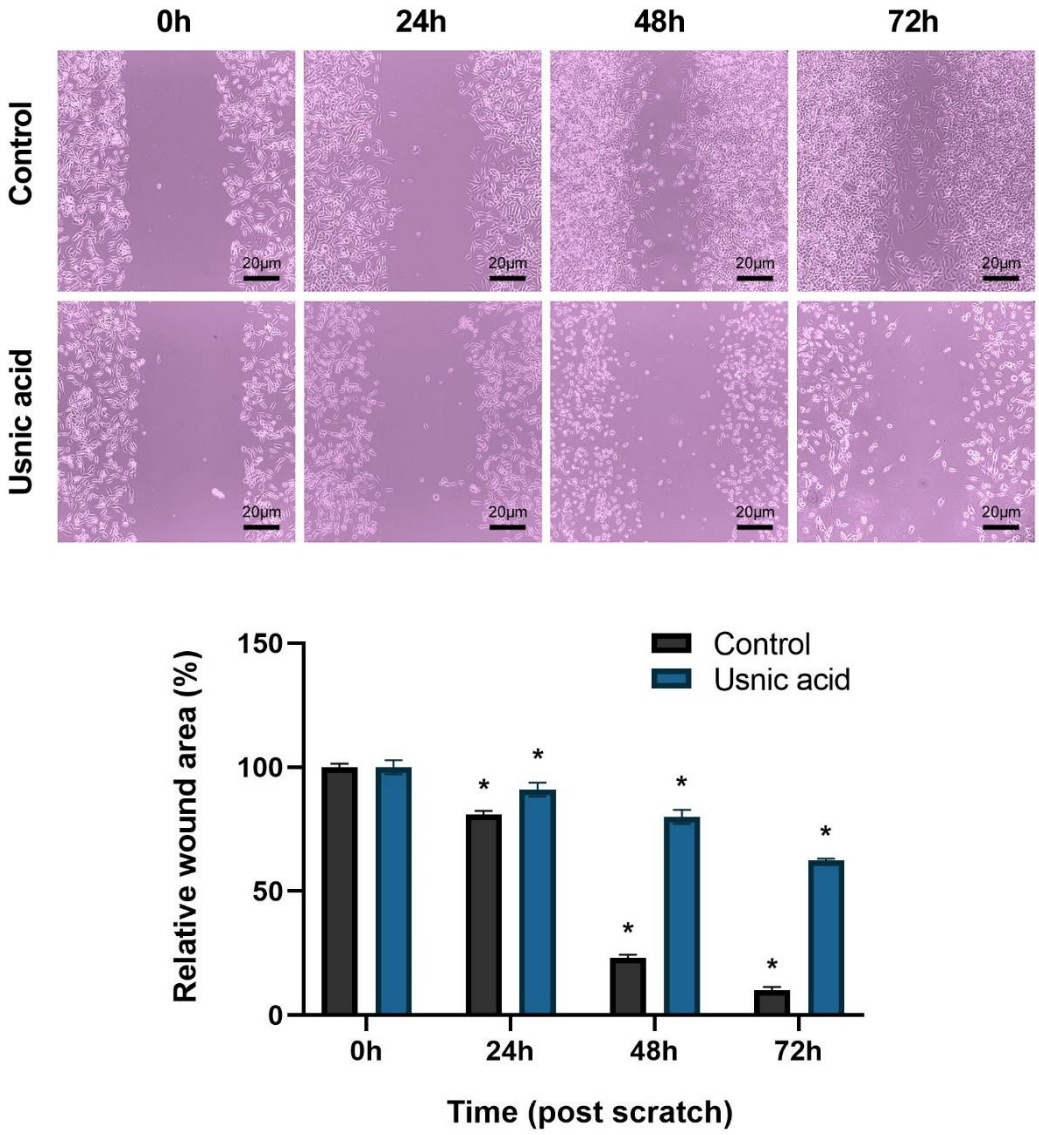

Figure 3. *In vitro* scratch assay. The photos were taken 0, 24, 48, and 72 h. Relative wound area in SK-UT-1 cells treated with UA vs. control group. *p <0.05.

In a study, it was reported that UA may have triggered the reactive oxygen species-dependent mitochondrial pathway-mediated apoptotic mechanism [22]. In a recent study, UA significantly reduced cell proliferation in AGS gastric cancer cells in the dose range of 10-50 µM and induced apoptosis in gastric cancer cells [17]. Galanty et al. (2017) demonstrated that UA exhibits anti-proliferative effects on DU145 and PC3 prostate cancer under *in vitro* conditions [18]. Wu et al. [23] have showed that UA inhibits cell proliferation in colorectal cancer cells through ATM-mediated DNA damage signaling pathway. Furthermore, they have demonstrated that UA inhibited colorectal cancer cell migration in the cell culture wound healing assay model.

In another study, it was shown that UA causes inhibition of cell motility in non-small cell lung cancer cells [24]. In a study, when the cytotoxic effects of UA on the HepG2 cell line, NS20Y, and HUVEC cells were analyzed, it was reported that the IC50 value of UA was higher in HUVEC cells used as control cells compared to cancer cells [25]. Emsen et al. [26] have showed that the UA decreased cell viability in U87MG glioblastoma cells and primary rat cerebral cortex (PRCC) cells. While the IC50 value in glioblastoma cells was determined as 41.55 mg/L, the IC50 value in PRCC cells was determined as 132.69 mg/L, which is a higher dose than glioblastoma. Similar to our study, it was reported that

UA showed more cytotoxic activity in cancer cells in comparison with non-cancerous cell lines [18, 27-29].

It was demonstrated by *in vitro* clonogenic test that UA inhibits colony formation in A549 cells. It was showed that UA treatment not only reduces the colony amount of A549 lung cancer but also reduces the colony sizes, especially depending on the increasing dose [30]. In a study on the anticancer activity of UA in COLO-205 colon cancer cells, it was shown that UA inhibited colony formation even at low doses, as well as inhibited cell proliferation [31]. Our results showed that the UA has a cytotoxic effect on the SK-UT-1 cells. The UA was cytotoxic after 48 h at concentrations above 5 μM, decreasing the viability of SK-UT-1 cells to about 13% at the highest concentration (25 μM). Besides, UA significantly revealed less toxicity on non-cancerous (HEK293) cells in comparison with the SK-UT-1 cells, HEK293 viability at 25 μM did not decrease below 40%. In the colony formation assay, we found that UA significantly inhibited the colony formation capability for SK-UT-1 cells. Similar effects were also observed in a wound-healing assay.

4. Conclusion

Our results suggest that UA can be an alternative bioactive agent for human uterine LMS SK-UT-1 (also known as HTB-114) cells. Our study is the first examination of the antiproliferative effects of the UA on SK-UT-1 cells. The molecular mechanism underlying the potential anti-cancer activities of UA should be investigated in further studies.

Authorship contribution statement

D. Mutlu: Methodology, Visualization, Original Draft Writing; **M. Seçme**: Methodology, Advice; **Ş. Arslan**: Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Committee Approval and/or Informed Consent Information

As the authors of this study, we declare that we do not have any ethics committee approval and/or informed consent statement.

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