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# Anti-angiogenic and antiproliferative properties of the lichen substances (-)-usnic acid and vulpinic acid

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**Abstract:** The anti-proliferative activities of the lichen substances (-)-usnic acid and vulpinic acid on the viability of HepG2 hepatocarcinoma cells, NS20Y neuroblastoma cells and HUVEC endothelial cells were studied by the MTT assay. The anti-angiogenic potential of the substances was determined by the endothelial tube formation assay. Both lichen substances exhibited strong anti-angiogenic activity and were more cytotoxic to the cancer cell lines than to the normal cell line, but vulpinic acid has more potential as an anti-angiogenic substance because of its low cytotoxicity and stronger anti-angiogenic activity on the HUVEC cell line.

**Keywords:** *Cladonia foliacea; Letharia vulpina*; (-)-usnic acid; vulpinic acid.

## 1 Introduction

Angiogenesis, the formation of new blood vessels from existing vasculature, plays essential roles in tumor invasion and metastasis [1]. Newly generated vessels also play a critical role in tumor development by supplying oxygen and nutrients and by removing waste products [2]. Angiogenesis inhibition is therefore an attractive strategy to prevent development, invasion and metastasis of tumors.

Herbal medicine has been used in a wide range of cancer treatments for thousands of years and still remains a very significant source of biologically active compounds [3]. Lichens are structurally complex symbiotic organisms composed of mycobiont (fungus) and photobiont (algae and/or cyanobacteria) organisms. Certain lichen secondary compounds (ca. 1000 presently known) derived from fungal metabolism have antimicrobial, antiviral, antiprotozoal, antiproliferative, antitumor, anti-inflammatory, anti-pyretic, analgesic, photoprotective, enzyme inhibitory and anti-angiogenic properties [4–8]. Usnic acid, a dibenzofuran derivative, is one of the most frequent and most studied lichen metabolites [9]. It is found commonly in many lichen species [5, 10]. Where it occurs in two chiral forms, i.e. as (*R*)-(+)-usnic acid and (*S*)-(-)-usnic acid [11], which differ in their bioactivities. For example, Romagni et al. [12] reported that (-)-usnic acid inhibits plant *p*-hydroxyphenylpyruvate dioxygenase more strongly by an order of magnitude than the (+)-enantiomer. Usnic acid displays in vitro antiproliferative activity against a wide variety of human and murine cancer cell lines [13, 14]. The toxicity of usnic acid has been related to induction of apoptosis in L1210 (murine leukemia) and A549 (human lung carcinoma) cells [15–17], to mitochondrial dysfunction in A549, T-47D (breast cancer), Capan-2 (pancreatic cancer) and HepG2 (human hepatoblastoma) cell lines [17–19] and with increased cytochrome P450 activity and/or oxidative stress in HepG2 and SH-SY5Y (human neuroblastoma) cell lines [19, 20]. In addition, Song et al. [7] reported that (+)-usnic acid inhibited angiogenesis by suppressing the VEGFR2-mediated downstream AKT and ERK1/2 signaling pathways. Although there is a considerable number of publications on the bioactivities of (+)-usnic acid [7, 10, 17], (-)-usnic acid has been poorly studied in comparison [11, 14, 16].

Vulpinic acid (Figure 1B) is a pulvinic acid derivative which has also been poorly investigated. Vulpinic acid was reported to be less toxic than (+)-usnic acid to MM98 (malignant mesothelioma), A431 (vulvar carcinoma), and HaCaT (human keratinocyte) cell lines [21], while both compounds induce uncoupling of mitochondrial oxidative phosphorylation.

The purpose of the present study was to investigate the cytotoxic activity of (-)-usnic and vulpinic acids against HepG2 (human hepatocarcinoma), NS20Y (mouse neuroblastoma), and HUVEC (human umbilical vein endothelial) cell lines. To the best of our knowledge, this is the first study on the anti-angiogenic properties of (-)-usnic acid

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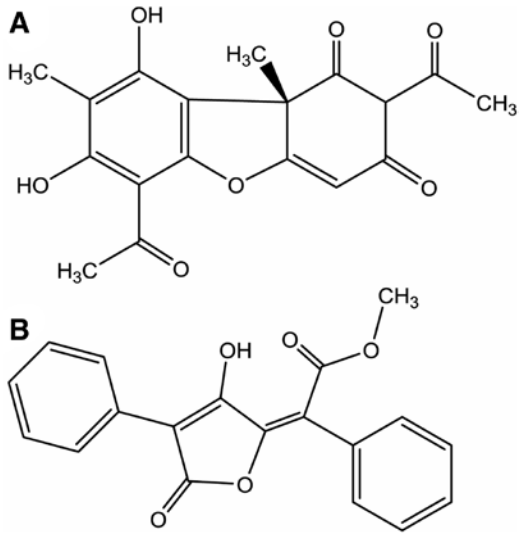


**Figure 1:** The chemical structures of (-)-usnic acid (A) and vulpinic acid (B).

and vulpinic acid. The observations suggest that (-)-usnic and vulpinic acids could be potential anti-angiogenic agents for antitumor treatment.

# 2 Materials and methods

### 2.1 Lichen material and isolation of the substances

The procedures for obtaining the lichens and lichen substances have been previously described [11, 22]. Briefly, (-)-usnic acid and vulpinic acid were isolated from the acetone extracts of the lichens *Cladonia foliacea* (Huds.) Willd and *Letharia vulpina* (L.) Hue, respectively. *Cladonia foliacea* was collected east of Mayıslar village, Eskisehir Province, Turkey, at 230 m above sea level. *L. vulpina* was collected from Bozdağ, Eskisehir Province, Turkey, at 1200 m. Herbarium samples of the lichen materials have been deposited at the Herbarium of Anadolu University in the Department of Biology (ANES). Stock solutions of the lichen substances were initially prepared in DMSO at 0.05–0.1 M concentration and further diluted in fresh complete medium.

### 2.2 Cell culture materials and conditions

Dulbecco's modified eagle's medium (DMEM), nutrient mixture Ham's F-12 K medium, endothelial cell growth supplement (ECGS), fetal bovine serum (FBS), penicillin-streptomycin, MTT, and matrigel were obtained from Sigma-Aldrich (St. Louis, MO, USA). Endothelial cell basal medium-2 (EBM-2) was purchased from Cambrex Bio Sciences (Walkersville, MD, USA). Tissue culture plates were purchased from TPP (Trasadingen, Switzerland). The HUVEC cell line (ATCC CRL-1730) was obtained from ATCC (American Type Culture Collection), HepG2 cells (ATCC HB-8065) were a kind gift of Hülya Sivas (University of Anadolu, Eskisehir, Turkey), and NS20Y cells (Sigma 08062517) were a kind gift of Emel Ergene (University of Anadolu). HUVECs were cultivated as a monolayer in nutrient mixture Ham's F-12 K containing ECGS, 20% heat inactivated-FBS, 1% penicillin-streptomycin and sodium bicarbonate. HepG2 and NS20Y cell lines were cultivated as monolayers in DMEM containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, and sodium bicarbonate, at 37 °C in a 5% $CO_2$ humidified incubator.

### 2.3 Cell viability assay

Growth inhibitory activities of (-)-usnic acid and vulpinic acid towards HepG2 ($8\times10^3$ cells/well), NS20Y ($5\times10^3$ cells/well), and HUVEC ($8\times10^3$ cells/well) cell lines were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [23]. Briefly, cells were seeded in 96-well culture plates and, after 24 h incubation, treated for 48 h with different concentrations of the test substances. Eight replicate wells were used for each concentration, and three independent experiments were performed at different intervals. Absorbance of the produced formazan was measured at 570 nm by a Bio-Tek ELX808IU microplate reader (Winooski, VT, USA).

### 2.4 Matrigel tube formation assay

Endothelial tube formation assays were performed as previously described [24]. HUVECs were serum starved by culturing in EBM-2 containing 2% FBS for 4 h. Then, HUVECs ($4\times10^4$ cells/well) were plated on matrigel coating the wells of 96-well plates, and were equilibrated with EBM-2 medium containing the substances. After 12 h incubation, tube formation was observed under an Olympus IX71 inverted microscope (Tokyo, Japan) and photographed with an Olympus DP70 camera (Tokyo, Japan) at 10× magnification.

### 2.5 Statistical analysis

The data obtained from the MTT assays were evaluated by one-way ANOVA followed by Tukey's test in SPSS (statistical package for social sciences). Results were expressed as percentages of the control as the mean ± SD, and differences from the control groups were considered significant with $p<0.05$. In addition, $IC_{50}$ values of the substances were calculated by nonlinear regression analysis of at least three separate triplicate experiments by the software 'Helper of Cell Culture Lab. v.1' of Mehmet Varol [25].

# 3 Results

## 3.1 Cell viability

(-)-Usnic acid and vulpinic acid reduced the viability of HUVEC, HepG2, and NS20Y cells in a time- and concentration-dependent manner (Figures 2, 3). The respective

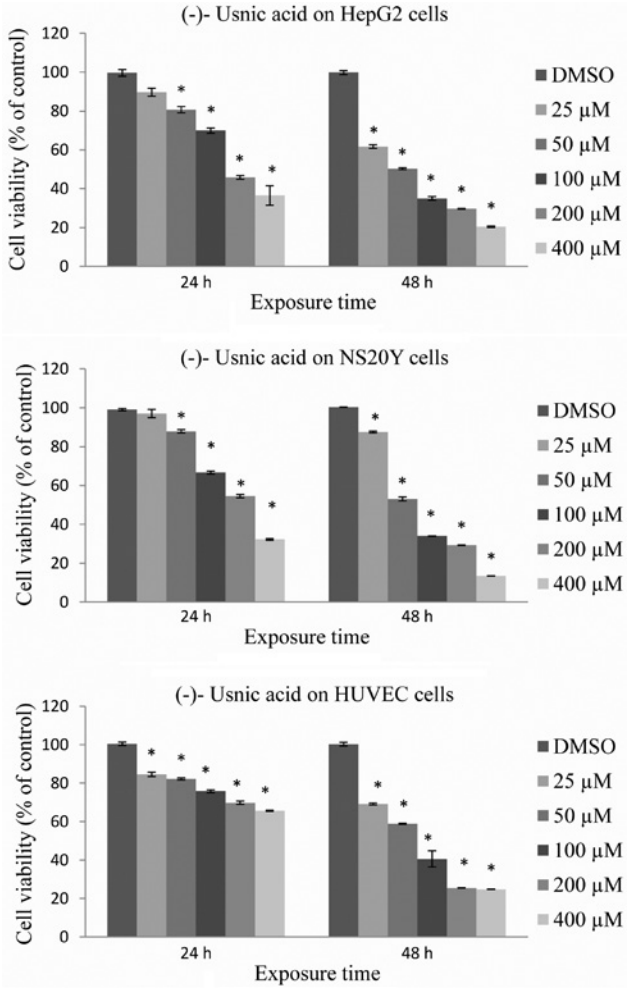


**Figure 2:** Antiproliferative activity of (-)-usnic acid on HepG2, NS20Y, and HUVEC cell lines.

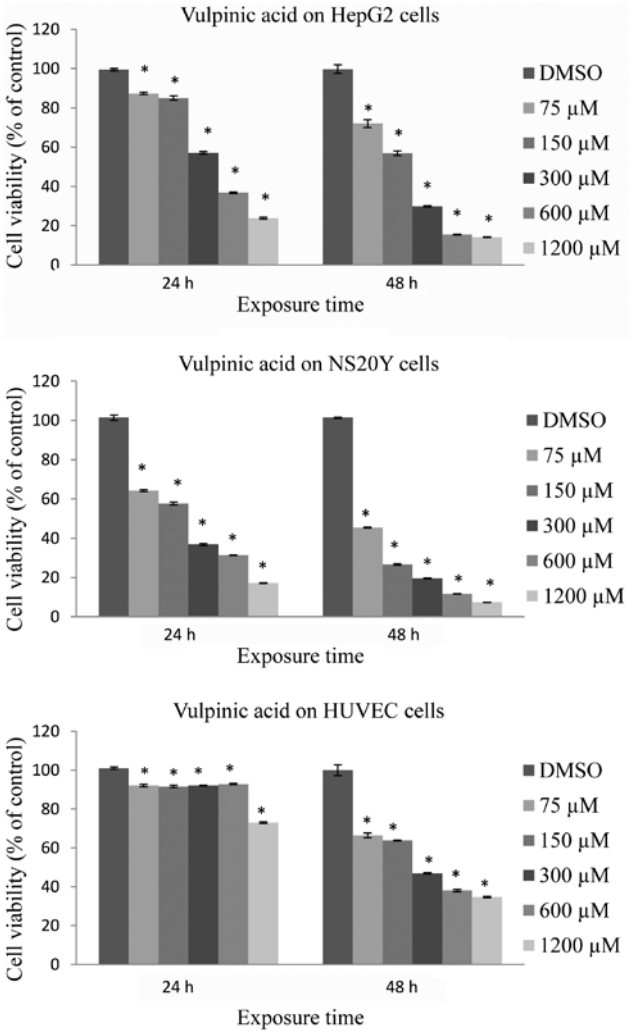


**Figure 3:** Antiproliferative activity of vulpinic acid on HepG2, NS20Y, and HUVEC cell lines.

half-maximal inhibitory concentrations ($IC_{50}$) are given in Table 1. (-)-Usnic acid was found to be more cytotoxic towards all cell lines than vulpinic acid. While (-)-usnic acid exhibited the strongest anti-proliferative effect on the HepG2 cell line, NS20Y cells were most affected by vulpinic acid. Of further interest, both lichen substances were only mildly cytotoxic against HUVEC cells (Figures 2, 3), and a clear dose-dependence was obvious only at 48 h.

## 3.2 Angiogenesis

To determine the anti-angiogenic effects of the lichen substances, the endothelial tube formation assay was performed. Tube formation by endothelial cells is widely accepted as a key step in angiogenesis. HUVECs on matrigel form a tube-like network within 12 h [7]. HUVEC cells were exposed to the two lichen substances at concentrations below their respective $IC_{50}$ values of the 24 h

**Table 1:** $IC_{50}$ values (µM) of cell viability inhibition by (-)-usnic acid and vulpinic acid (±SD; n=3).

| Lichen substances | $IC_{50}$ values on HepG2 | | $IC_{50}$ values on NS20Y | | $IC_{50}$ values on HUVECs | |
|---|---|---|---|---|---|---|
| | 24 h | 48 h | 24 h | 48 h | 24 h | 48 h |
| (-)-Usnic acid | 160.6±4.38 | 50.24±1.23 | 217.31±3.51 | 52.18±1.71 | 427.9±1.15 | 71.5±0.19 |
| Vulpinic acid | 356.22±4.02 | 164.27±3.33 | 176.33±2.76 | 68.83±1.58 | 1890.51±3.56 | 231.94±25.4 |

treatment. Both (-)-usnic acid and vulpinic acid suppressed endothelial tube formation in a dose-dependent manner (Figure 4), (-)-usnic acid being the more active substance.

## 4 Discussion

The cell viability assays revealed that usnic acid has a higher antiproliferative potential than vulpinic acid, in agreement with previous data [21]. Of further interest, both lichen substances were less toxic to the HUVEC cell line than to the cancer cell lines (Table 1), in agreement with our previous results for the cytotoxicity of usnic acid to cancer cells (A549) and normal cells (V79). Although there are many studies on usnic acid, there is only one previous in vitro study on vulpinic acid [21], which revealed that vulpinic acid was more toxic to normal keratinocytes (HaCaT) than to MM98 malignant mesothelioma cells and A543 vulvar carcinoma cells. However, we found that vulpinic acid was significantly less toxic to HUVECs as compared to HepG2 and NS20Y cancer cell lines (Table 1).

Medicinal plants and phytochemicals with anti-angiogenic activity and low toxicity have gained importance. Compounds that are anti-angiogenic but not toxic are beneficial in combating cancer by preventing the formation of new blood vessels that support tumor growth [26]. Endothelial cells form blood vessels, and many

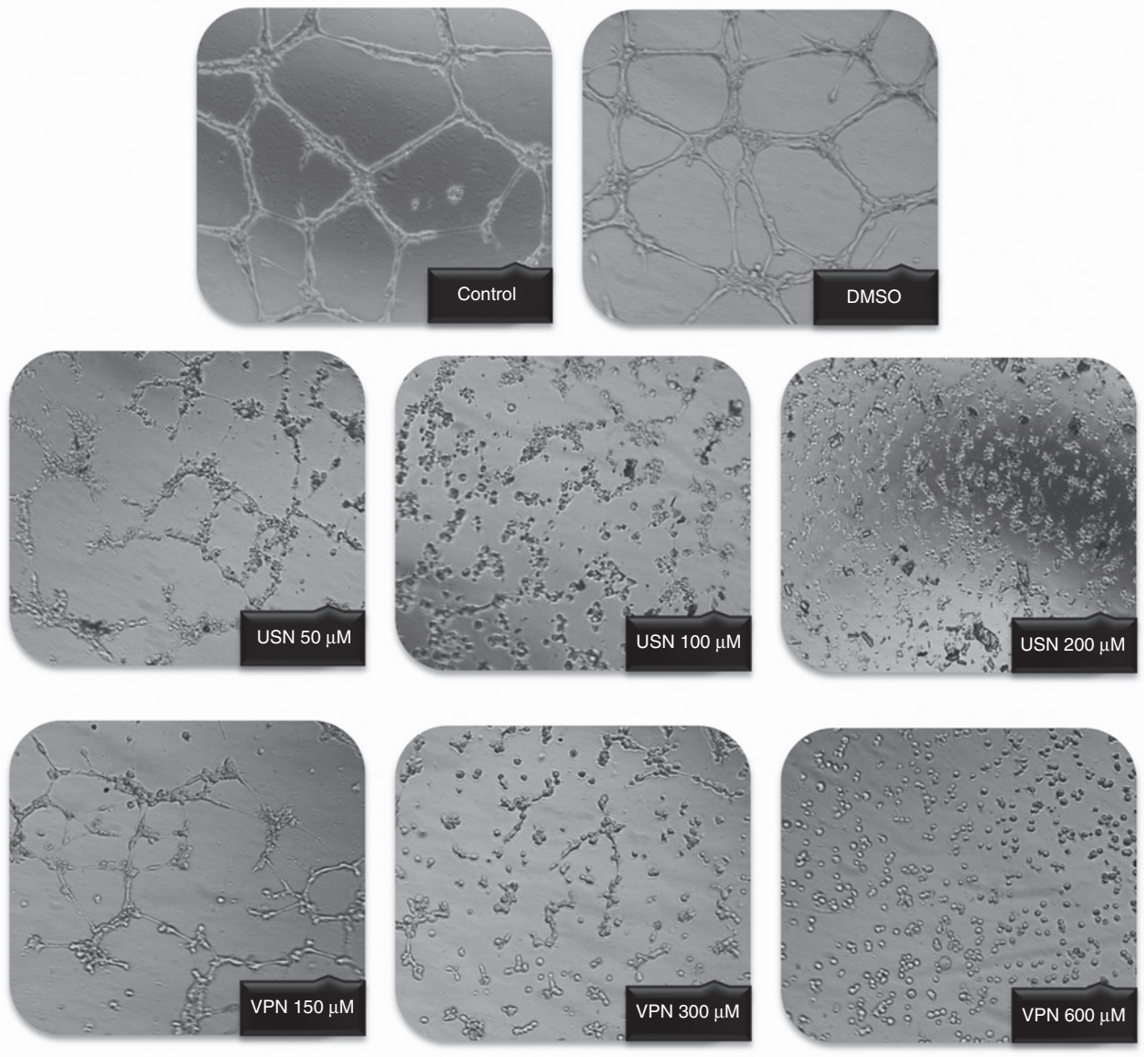


**Figure 4:** Effects of (-)-usnic acid (USN) and vulpinic acid (VPN) on human umbilical vein endothelial cell (HUVEC) tube formation.

anti-angiogenic substances are directed against their function [27]. The $IC_{50}$ value of the effect of (-)-usnic acid on HUVECs at 24 h was about 428 μM and that of vulpinic acid about 1890 μM (Table 1), while endothelial tube formation was already affected at much lower concentrations (Figure 4). Considering that tube formation was assessed after 12 h, cell viability should be affected even less at this time. Thus, endothelial tube formation by the HUVEC cells was far more sensitive to the lichen substances than their viability (Figure 4, Table 1), and vulpinic more favourably combined inhibitory activity on tube formation with low cytotoxicity.

Song et al. [7] extensively studied the anti-angiogenic activity of (+)- usnic acid and reported that (+)- usnic acid reduces the viability of Bcap-37 breast cancer cells more effectively as compared to HUVECs, in agreement with our data (Table 1). They reported that (+)- usnic acid inhibits VEGF-induced HUVEC migration and functions as an anti-angiogenic substance by suppressing VEGFR2-mediated AKT and ERK1/2 signaling pathways in a dose-dependent manner, as we did in our study.

In conclusion, this study revealed the anti-angiogenic activities of (-)-usnic acid and vulpinic acid. Both substances were more cytotoxic to HepG2 and NS20Y cancer cell lines than to normal HUVECs, and they both show strong anti-angiogenic potential. Thalidomide has been used to inhibit angiogenesis. Bostancioglu et al. [28] reported that a concentration of thalidomide as high as 300 μM was required to inhibit tube formation. Thus, (-)-usnic acid at 50 μM and vulpinic acid at 150 μM are more potent than thalidomide. Vulpinic acid seems to be more suitable as an anti-angiogenic substance than (-)-usnic acid, due to its lower toxicity and stronger anti-angiogenic activity. To understand the underlying mechanisms of anti-angiogenic activities of (-)-usnic acid and vulpinic acid, the substances should be studied extensively in in vitro and in vivo experimental models. Considering the obtained data, both lichen acids appear to be promising for the development of anti-angiogenic and hence anti-cancer drugs.

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