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RESEARCH

# Antiproliferative, antimigratory, and apoptotic effects of diffractaic and vulpinic acids as thioredoxin reductase 1 inhibitors on cervical cancer

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## Abstract

Cervical cancer is among the most frequently observed cancer types in females. New therapeutic targets are needed because of the side impacts of existing cancer drugs and the inadequacy of treatment methods. Thioredoxin reductase 1 (TrxR1) is often overexpressed in many cancer cells, and targeting TrxR1 has become an attractive target for cancer therapy. This study investigated the anticancer impacts of diffractaic and vulpinic acids, lichen secondary metabolites, on the cervical cancer HeLa cell line. XTT findings demonstrated showed that diffractaic and vulpinic acids suppressed the proliferation of HeLa cells in a dose- and time-dependent manner and $IC_{50}$ values were 22.52 µg/ml and 66.53 µg/ml at 48 h, respectively. Each of these lichen metabolites significantly suppressed migration. Diffractaic acid showed an increase in both the *BAX/BCL2* ratio by qPCR analysis and the apoptotic cell population via flow cytometry analysis on HeLa cells. Concerning vulpinic acid, although it decreased the *BAX/BCL2* ratio in this cells, it increased apoptotic cells according to the flow cytometry analysis results. Diffractaic and vulpinic acids significantly suppressed TrxR1 enzyme activity rather than the gene and protein expression levels in HeLa cells. This research demonstrated for the first time, that targeting TrxR1 with diffractaic and vulpinic acids was an effective therapeutic strategy for treating cervical cancer.

**Keywords** Diffractaic acid · Vulpinic acid · Thioredoxin reductase 1 · Anticancer potential · Cervical cancer

## Introduction

Cervical cancer takes place among the most frequent cancer types in females and ranks fourth in the world in cancer-related deaths (Mattiuzzi and Lippi 2019; Sung et al. 2021). It is mainly treated with surgery, chemotherapy, and radiotherapy treatment options (Narayan and Lin 2015). Novel therapeutic strategies and novel adjuvant agents are of extreme importance because of the side impacts of existing cancer drugs and inadequate treatment methods. The fact that natural products or their derivatives cover 16% of drugs, which received approval from the U.S. Food and Drug Administration in 2018 underlines the relevance of natural products to current drug discovery (de la Torre and Albericio 2019).

Lichens, which are natural products, consist of fungi that form a symbiotic relationship with green algae or cyanobacteria (Stocker-Wörgötter 2008). About 1000 secondary metabolites of lichens are known (Roser et al. 2022). Many biological properties of lichen secondary metabolites, e.g., antihepatotoxic, antimicrobial, antiviral, anti-inflammatory, antiangiogenic, and anticancer have been identified (Oettl et al. 2013; Solárová et al. 2020; Ulus 2021; Kalın et al. 2023). Numerous researchers have focused on lichen secondary metabolites because of these properties (Koparal 2015; Ozgencli et al. 2019; Ulus 2021; Kalın et al. 2022a; Günaydın et al. 2023).

Redox homeostasis, which is regulated by two main cellular antioxidant systems, the glutathione and thioredoxin systems, takes an essential part in cellular viability and function (Powis et al. 1997; Sun and Rigas 2008; Yin et al. 2012). Many cellular processes cause reactive oxygen

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species (ROS) to be formed as by-products (Ray et al. 2012). Cancer cells are the result of the activation of oncogenes and/or the inactivation of tumour suppressors. An important characteristic of cancer cells is their ability to grow indefinitely (Hanahan and Weinberg 2000). Since the production of ROS is a by-product of cell growth, cancer cells maintain a much higher level of ROS production than normal cells (Trachootham et al. 2009; Cairns et al. 2011; Moloney and Cotter 2018). To avoid the harmful effects of oxidative stress, cancer cells actively upregulate multiple antioxidant systems. By buffering ROS levels, cancer cells are able to limit ROS to a level that is favorable for tumour progression. The intracellular redox balance is disrupted by the amount of ROS reaching toxic levels, exerting oxidative stress on cancer cells which may lead to their death (Nogueira and Hay 2013).

The thioredoxin system, comprising thioredoxin reductase (TrxR), thioredoxin (Trx), and nicotinamide adenine dinucleotide phosphate (NADPH), takes an essential part in redox signaling for cell growth and apoptosis and ensures cell protection against oxidative stress (Yan et al. 2012; Ouyang et al. 2018; Bjørklund et al. 2022). However, TrxR1 overexpressed in many cancer cells facilitates proliferation, apoptosis resistance, angiogenesis, and metastasis (Lincoln et al. 2003, 2010; Branco et al. 2014; Shang et al. 2019; Mohammadi et al. 2019). Thus, targeting TrxR1 in cancer cells is stressed as a potential target for cancer therapy, given that it suppresses tumour progression and metastasis (Biaglow and Miller 2005; Zhang et al. 2017).

There are few studies on diffractaic and vulpinic acids, lichen secondary metabolites in different cancer types. However, the effects of these metabolites on human cervical cancer remain unclear. This study aimed to investigate whether diffractaic and vulpinic acids had pharmacological potential as agents for human cervical cancer treatment. To this end, it was analyzed whether they play an anticancer role mediated by TrxR1 by evaluating the affects of diffractaic and vulpinic acids on viability, migration, and apoptosis induction in human cervical cancer HeLa cell line.

## Materials and methods

### Cell culture and reagents

The human cervix carcinoma cell line (HeLa) was purchased from the ATCC (American Type Culture Collection, LGC Promochem, UK). HeLa cells were cultivated in RPMI 1640 medium (Sigma-Aldrich). There was 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone), 1% L-glutamine (Thermo Fisher Scientific), and 1% penicillin/streptomycin (Sigma-Aldrich) in this medium. The above-mentioned cell lines were cultured at a temperature of 37 °C in a 5% $CO_2$ atmosphere.

Diffractaic acid ($C_{20}H_{22}O_7$, TargetMol Chemicals Inc., Boston, MA, USA) and vulpinic acid ($C_{19}H_{14}O_5$, Cayman Chemical Company, Michigan, USA) were purchased, dissolved in dimethyl sulfoxide (DMSO), and stored at a temperature of -20 °C until being utilized.

### Cell viability assay

The cell viability assay was carried out as specified before (Kalın et al. 2022b). In brief, the XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay (Cell Proliferation Kit, Roche) was utilized with the objective of determining cell viability. The seeding of HeLa cells ($1 \times 10^4$ cells per well) was performed into 96-well plates. Various concentrations (10, 25, 50, 75 and 100 µg/ml) of diffractaic and vulpinic acids were added to the wells. The XTT assay evaluated the cells incubated with these lichen metabolites in a dose- and time-dependent (24 and 48 h) manner. Following incubation, absorbance measurement was performed at 470 nm in an Epoch microplate reader (BioTek, USA). The $IC_{50}$ values (inhibitory concentration 50%) were calculated by logarithmic conversion of the absorbance values, and all values represent the standard deviation (± SD) of three independent experiments.

### Transwell migration assay

Migration assay was performed in Transwell cell culture chambers (Millicell® Hanging Cell Culture Insert, Millipore, U.S.) with 8-µm pores. The cells ($5 \times 10^4$ cells/well) treated separately with the most effective $IC_{50}$ dose of diffractaic acid and vulpinic acid, as determined by cell viability analysis, were suspended in 250 µl of serum-free medium and added to the upper chamber of the Transwell insert (24-well plate). The lower chambers were filled with 500 µl of RPMI containing 10% FBS. The cell incubation was carried out at a temperature of 37 °C for a period of 24 h in a 5% $CO_2$ atmosphere. The cells migrating to the opposite side of the Transwell membrane were fixed with methanol. They were then stained with a 0.3% solution of crystal violet and were counted under an inverted light microscope (Zeiss Primovert, Germany). All measurements were made in triplicate for every cell line, and image analyzer (ImageJ2x software) was utilized for the purpose of measuring the percentage of six random fields (Barlak et al. 2020).

### Cell apoptosis analysis

The apoptosis assay was carried out on HeLa cells, as described before (Altay et al. 2022). Briefly, the cells

($3 \times 10^5$ cells/well) were seeded into six-well plates, and their incubation was performed for a night at a temperature of 37 °C in a 5% $CO_2$ atmosphere. The cells treated with diffractaic and vulpinic acids ($IC_{50}$ values) were incubated for 48 h. Moreover, hydrogen peroxide ($H_2O_2$, 100 µM) was utilized as a positive control. Post-incubation steps were performed using the Annexin V-FITC/propidium iodide (PI) double staining detection kit following the instruction manual (BioLegend, San Diego, CA). The untreated cells were utilized as a negative control group. Flow cytometry (Beckman Coulter CytoFLEX, Brea, CA) was used for sample detection. The results are given as means ± SD of three independent experiments.

## cDNA synthesis and quantitative real-time PCR (qPCR)

HeLa cells were treated with the most effective $IC_{50}$ dose of each of diffractaic acid and vulpinic acid for 48 h. The extraction of total RNA from the cultured cells was performed with the RNA isolation PureLink™ RNA Mini Kit (Invitrogen) in line with the protocol of the manufacturer. A high-capacity cDNA reverse transcription kit (Applied Biosystems) was utilized for the synthesis of the first-strand cDNA in line with the manufacturer's protocol. The qPCR analysis of Bcl2 apoptosis regulator (*BCL2*), Bcl2 associated X, apoptosis regulator (*BAX*), and *TrxR1* target gene expressions was carried out by utilizing SYBR Green master mix (BioRad) with Rotor-Gene Q (Qiagen). As a housekeeping gene, β-actin was used. Amplification curve analysis was conducted to measure the products amplified. The analysis of all data was performed by employing the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

## Western blot analysis

HeLa cells were incubated with $IC_{50}$ concentrations of diffractaic and vulpinic acids for a period of 48 h. The cells were harvested and lysed in 400 µl of the radioimmunoprecipitation assay buffer (RIPA buffer, 9806, Cell Signaling Technology), containing 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich). The centrifugation of the homogenate was carried out, and the supernatants were collected and stored at a temperature of − 80 °C. The Bradford protein assay was used for the assessment of protein concentrations. The total protein extract (40 µg) was separated in the electrophoretic field and then the proteins in the gel were transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking with 5% skim milk powder (Sigma-Aldrich) at 1 h, membrane incubation was carried out for a night at a temperature of 4 °C using a specific primer TrxR1 antibody (Santa Cruz Biotechnology, sc-28321, 1:1000) and β-actin antibody (Santa Cruz Biotechnology, sc-47778, 1:1000). Then, the membranes were washed and their incubation was carried out at room temperature for 1 h with horseradish-coupled secondary antibodies (Santa Cruz Biotechnology) at a 1:10,000 dilution. An enhanced chemiluminescence detection system (ECL Clarity/ ECL Clarity Max Substrate, Biorad) was employed for signal visualization. ImageJ2x was used to analyse the images obtained from Western blot analysis (Baltacı et al. 2022).

## Intracellular TrxR1 activity determination

TrxR1 enzyme activity was measured using the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method, which is based on the NADPH-dependent reduction of disulfide bonds in DTNB. To this end, the cells were harvested and lysed in 400 µl of RIPA buffer (9806, Cell Signaling Technology), which contained 1 mM PMSF (Sigma-Aldrich). Two hundred microliter of 100 mM Potassium phosphate (Sigma-Aldrich) buffer (pH 7)/10 mM ethylene diamine tetra acetic acid (EDTA, Sigma-Aldrich) (pH 7), 100 µl of 0.2 mM NADPH (Sigma-Aldrich), 100 µl of 0.2 mg/ml bovine serum albumin (BSA, Sigma-Aldrich), 100 µl of 5 mM DTNB (Sigma-Aldrich), and 30 µl of supernatant were added to the test tube and the final volume was made up to 1000 µl with $dH_2O$. Changes in TrxR1 enzyme activity were monitored every 3 min at 412 nm using a UV–VIS spectrophotometer (Beckman Coulter, California, U.S.). The activity of TrxR1 is based on the detection of TNB (2-nitro-5-thiobenzoic acid), which results from the reduction of DTNB per minute. The molar extinction coefficient for TNB was 14.15 M(-1)cm(-1). The enzyme unit (EU) of TrxR1 activity was defined as the production of 1 micromolar TNB per minute under these conditions. The specific activity of TrxR1 was determined as EU/mg protein and expressed as a percentage of the control (Sönmez Aydın et al. 2021; Hukkamlı et al. 2023; Kalın et al. 2023).

## Statistical analysis

Data were acquired from three experiments and given as mean ± SD. The unpaired t-test and two-way analysis of variance (ANOVA) were conducted for the purpose of comparing the results statistically in the GraphPad Prism Software version 5.0 for Windows (GraphPad Software). A symbol (*) shows statistically significant alterations. The symbol expressions are given below: $p > 0.05$ (not significant, ns); $*p < 0.05$ (significant), $**p < 0.01$ (very significant), and $***p < 0.001$ (extremely significant).

## Results

### Diffractaic acid and vulpinic acid effectively suppressed the HeLa cell viability

To investigate the potential benefit of lichen secondary metabolites (diffractaic and vulpinic acids) in human cervical carcinoma, HeLa cells were treated using the XTT assay at low to high concentrations (10–100 µg/ml) of both lichen metabolites for 24 and 48 h. The microscopic image results in Fig. 1A demonstrated that the number of viable cells and the morphological shape of HeLa cells treated with diffractaic and vulpinic acids, changed significantly in a dose- and time-dependent manner. As shown in Fig. 1B, diffractaic acid did not display an effective inhibitory effect in HeLa cells up to 25 µl/ml for 24 h, but significantly reduced cell viability at doses of 50 µg/ml and above. Considering the time-dependent effect of diffractaic acid on HeLa cell viability, significant inhibition was observed up to 25 µg/ml at 48 h, and this inhibitory effect was more effective at increasing doses. The $IC_{50}$ values of diffractaic acid in HeLa cells were determined as 72.26 ± 2.72 µg/ml and 22.52 ± 2.24 µg/ml at 24 h and 48 h, respectively. Hence, the following steps of the current work were performed with the most effective $IC_{50}$ value of diffractaic acid determined at 48 h in the HeLa cell line.

The increasing concentrations of vulpinic acid remarkably decreased cell viability at both 24 h and 48 h. The $IC_{50}$ value of vulpinic acid in HeLa cells was computed as 64.28 ± 4.53 µg/ml and 66.53 ± 4.03 µg/ml at 24 h and 48 h, respectively (Fig. 1C). The $IC_{50}$ values of vulpinic acid in HeLa cells were close to each other at both times. However, the inhibitory effect of vulpinic acid was more regular at 48 h than at 24 h (up to a dose of 100 µg/ml). Our results showed that diffractaic acid was more cytotoxic than vulpinic acid in HeLa cells at 48 h. The $IC_{50}$ value at 48 h in HeLa cells was used in the later stages of the present study.

### Diffractaic and vulpinic acids exhibited antimigratory and apoptotic effects in HeLa cells

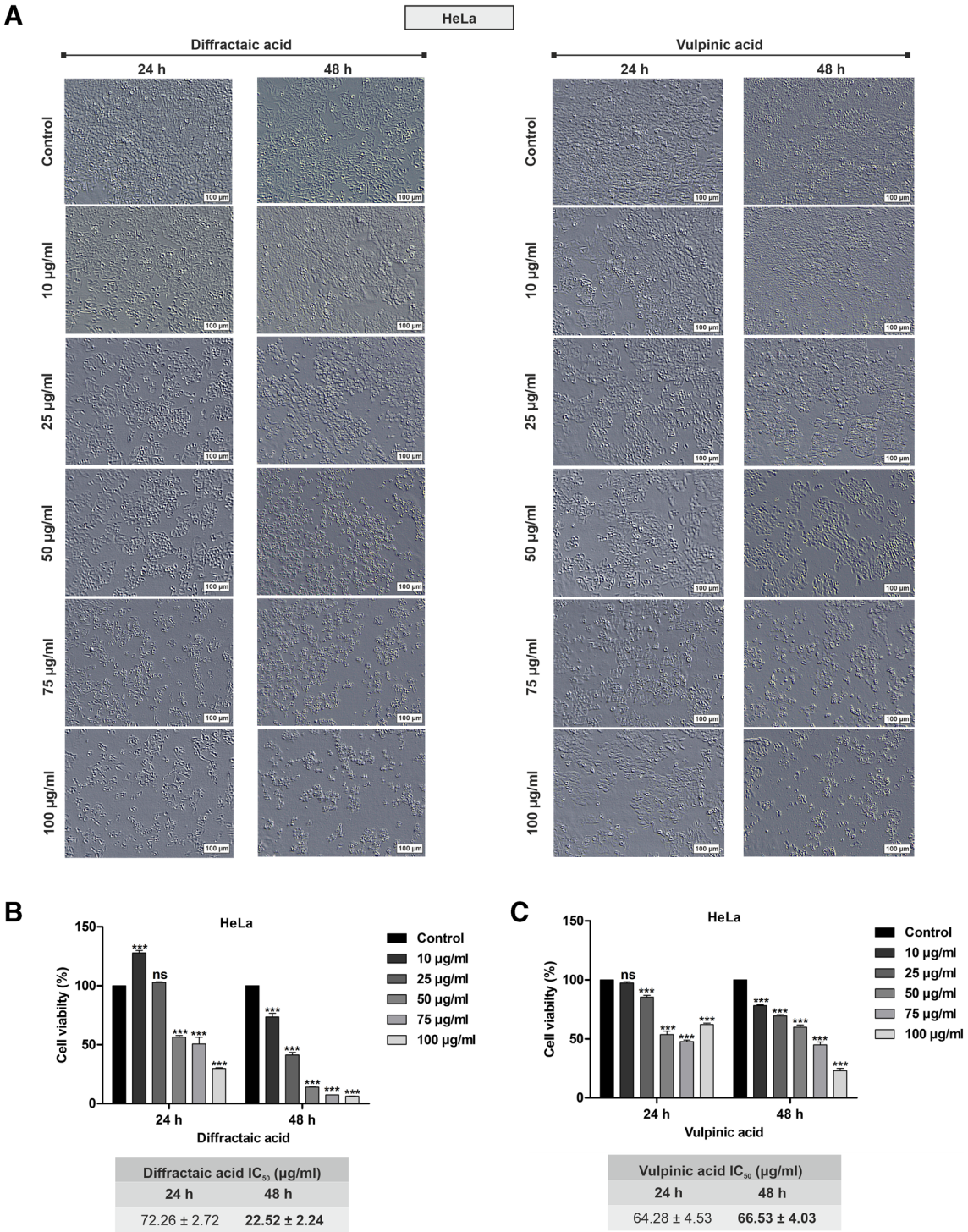
The present work was carried out for the purpose of evaluating whether both lichen secondary metabolites could suppress the ability of HeLa cells to migrate using transwell migration assays. Thus, diffractaic acid (22.52 ± 2.24 µg/ml) and vulpinic acid (66.53 ± 4.03 µg/ml) were treated with HeLa cells. The incubation time in the transwell migration assay was determined to be 24 h. The transwell migration test results showed that both diffractaic acid ($p < 0.01$) and vulpinic acid ($p < 0.001$) treatment considerably suppressed migration in HeLa cells (Fig. 2A). Our data showed that vulpinic acid had a stronger antimigratory effect than diffractaic acid.

For the further investigation of the potential mechanism of both secondary metabolites that inhibit the growth of HeLa cells, we also determined the apoptosis phenomenon with qPCR and flow cytometry analysis. As seen in Fig. 2B, the qPCR results showed increased *BAX* ($p < 0.01$) and *BCL-2* ($p < 0.001$) mRNA levels, and the *BAX/BCL2* ratio ($p < 0.01$) was also significantly elevated in the HeLa cells treated with diffractaic acid.

On the contrary, vulpinic acid suppressed the mRNA level of *BAX* ($p < 0.01$), increasing that of *BCL-2* ($p < 0.01$), which resulted in a decreased *BAX/BCL-2* ratio ($p < 0.001$) (Fig. 2C). Our findings revealed that *BAX*, a pro-apoptotic marker, was predominant in diffractaic acid-treated HeLa cells, whereas *BCL2*, an anti-apoptotic marker, was more dominant in those treated with vulpinic acid. In Fig. 2D, flow cytometry studies were carried out with the objective of confirming the qPCR results, and it was seen that both diffractaic and vulpinic acids increased the percentage of early apoptotic (from ~ 1.2% to ~ 5.0%, $p < 0.001$; from ~ 1.5% to ~ 8.1%, $p < 0.001$), late apoptotic (from ~ 3.4% to ~ 15.7%, $p < 0.001$; from ~ 3.9% to ~ 17.7%, $p < 0.001$), and necrotic cells (from ~ 3.2% to ~ 9.3%, $p < 0.001$; from ~ 2.8% to ~ 11.1%, $p < 0.001$) in HeLa cells compared to the control group, respectively. The positive control group was treated with $H_2O_2$ (100 µM), and it was determined that while cell viability decreased, the percentages of late apoptotic and necrotic cells increased significantly in the HeLa cells. Our results showed that both diffractaic acid and vulpinic acid predominantly induced apoptosis in HeLa cells.

### Lichen secondary metabolites targeted TrxR1 and inhibited its catalytic activity in vitro

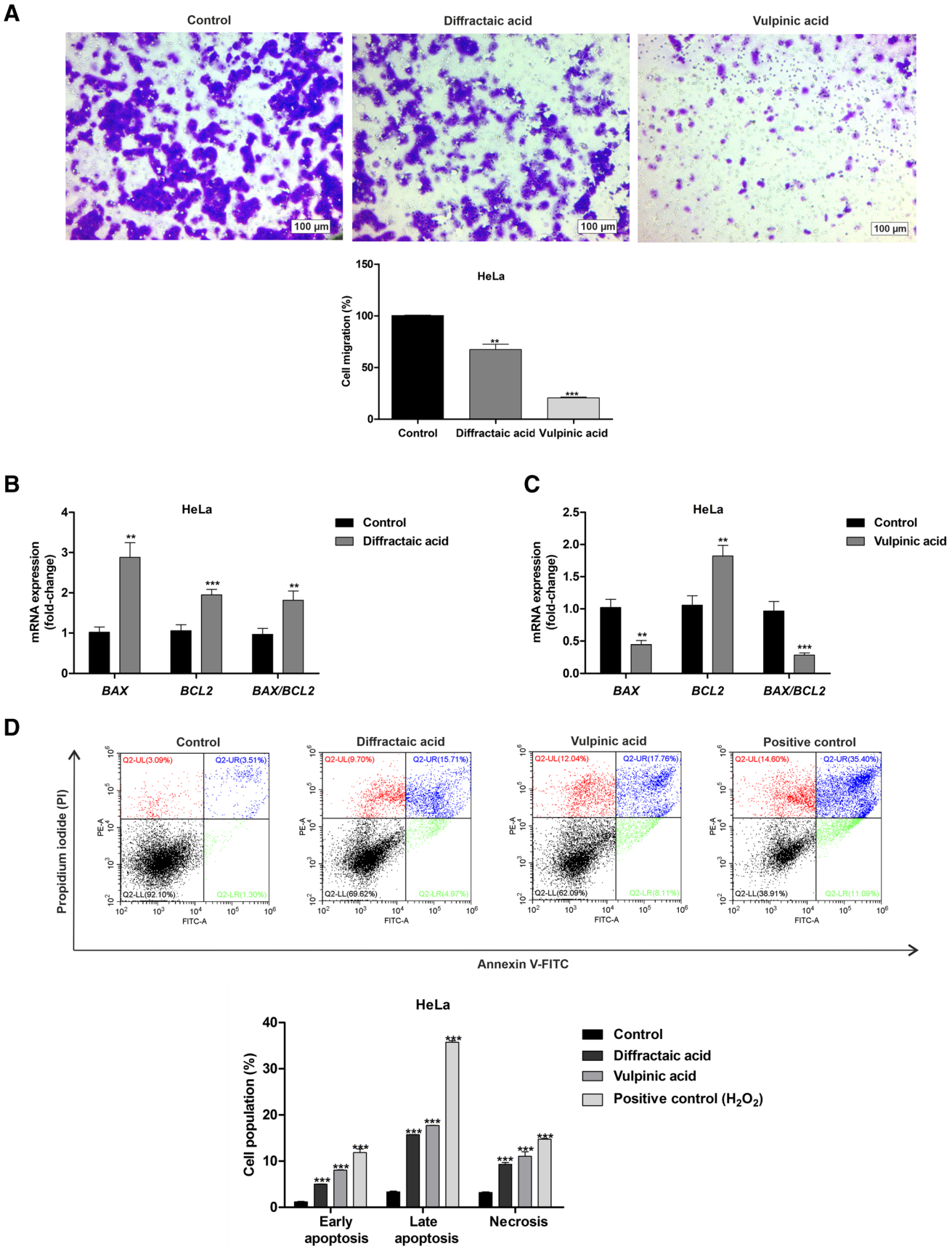
Diffractaic and vulpinic acids may exert antimigratory, antiproliferative, and apoptotic impacts through a potential target in HeLa cells. To this end, alterations in mRNA, protein, and enzymatic activity levels were researched to understand whether both lichen metabolites targeted TrxR1 on HeLa cells. The qPCR results showed that vulpinic acid significantly increased the mRNA level of *TrxR1* ($p < 0.001$), but diffractaic acid did not change it in the HeLa cell line (Fig. 3A-B). The impacts of both lichen metabolites on the protein expression levels of TrxR1 in HeLa cells were identified by western blot analysis, and the results demonstrated that both did not alter TrxR1 protein levels (Figs. 3C-D). The enzymatic activity of TrxR1 was significantly inhibited in HeLa cells treated with both diffractaic ($p < 0.001$) and vulpinic acids ($p < 0.001$) in comparison with control cells (Figs. 3E-F).




| Diffractaic acid $IC_{50}$ (µg/ml) | |
|---|---|
| 24 h | 48 h |
| 72.26 ± 2.72 | **22.52 ± 2.24** |

| Vulpinic acid $IC_{50}$ (µg/ml) | |
|---|---|
| 24 h | 48 h |
| 64.28 ± 4.53 | **66.53 ± 4.03** |

**Fig. 1** Effect of lichen secondary metabolites on cell viability in human cervical carcinoma. **A** Dose- and time-dependent microscope images of the HeLa cell line treated with diffractaic and vulpinic acids. **B-C** The antiproliferative effects and the $IC_{50}$ values (µg/ml) of diffractaic and vulpinic acids against HeLa cells. The experiment was conducted in three- biological and technical replicates. Scale bar, 100 µm. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ in relation to the control


A
Control
Diffractaic acid
Vulpinic acid
100 μm
HeLa
Cell migration (%)
Control
Diffractaic acid
Vulpinic acid
B
HeLa
mRNA expression (fold-change)
Control
Diffractaic acid
BAX
BCL2
BAX/BCL2
C
HeLa
mRNA expression (fold-change)
Control
Vulpinic acid
BAX
BCL2
BAX/BCL2
D
Control
Diffractaic acid
Vulpinic acid
Positive control
Q2-UL(3.09%)
Q2-UR(3.51%)
Q2-LL(92.10%)
Q2-LR(1.30%)
Q2-UL(9.70%)
Q2-UR(15.71%)
Q2-LL(69.62%)
Q2-LR(4.97%)
Q2-UL(12.04%)
Q2-UR(17.76%)
Q2-LL(62.09%)
Q2-LR(8.11%)
Q2-UL(14.60%)
Q2-UR(35.40%)
Q2-LL(38.91%)
Q2-LR(11.09%)
PE-A
FITC-A
Propidium iodide (PI)
Annexin V-FITC
HeLa
Cell population (%)
Early apoptosis
Late apoptosis
Necrosis
Control
Diffractaic acid
Vulpinic acid
Positive control (H2O2)

◂**Fig. 2** The anti-migratory and apoptotic effects of diffractaic and vulpinic acids in the HeLa cell line. **A** The migration potential of the cells treated with these lichen secondary metabolites was evaluated by the transwell migration assay. Images of cells migrating to the lower surface of the membrane were captured using a microscope camera. **B-C** Expressions of *BAX* and *BCL2* genes analyzed via qPCR in HeLa cells. **D** The percentage of apoptotic cells was determined in each group by Annexin-V/PI staining and flow cytometry. Early apoptosis was localized in the bottom right area, late apoptosis in the top right area, and necrosis in the top left area. The experiment was conducted in three- biological and technical replicates. Scale bar,100 µm. $*p<0.05$, $**p<0.01$, and $***p<0.001$ in relation to the control

## Discussion

Lichen secondary metabolites have significant pharmacological potential in cancer therapy (Dar et al. 2022). This situation reveals the importance of investigating natural or synthetic compounds with anticancer effects. In the current work, we first investigated the anti-cancer potential of diffractinic acid and vulpinic acid in the HeLa cell line in a dose- and time-dependent manner. The findings obtained indicated the sensitivity of HeLa cells to increased concentrations of diffractaic and vulpinic acids. We found $IC_{50}$ values of 22.52 µg/ml and 66.53 µg/ml for diffractaic and vulpinic acids on HeLa cells at 48 h, respectively. Thus, our data showed that diffractaic acid displayed stronger cytotoxicity than vulpinic acid in HeLa cells.

A low number of studies is available in the literature on the different cytotoxic impacts of both lichen acids in different cancer lines (Brisdelli et al. 2013; Kim et al. 2017; Kılıc et al. 2018). For example, a study reported that diffractaic acid significantly inhibited cell viability in human breast cancer MCF-7 and MDA-MB-453 cells with $IC_{50}$ values of 51.32 µg/ml and 87.03 µg/ml, respectively (Kalın et al. 2022a). Furthermore, diffractaic acid exerted a potent cytotoxic effect on HeLa, NCI-H460 (human lung cancer), and MCF-7 cell lines at 100 µg/ml (Truong et al. 2014). Concerning vulpinic acid, it was stated that it exhibited cytotoxic effects in MCF-7 and MDA-MB-453 cells with $IC_{50}$ values of 22.92 µg/ml and 95.65 µg/ml, respectively (Kalın et al. 2022b). Our results showed that diffractaic acid had a more potent antiproliferative effect in HeLa cells than reported in the literature. In addition, the cytotoxic effect of vulpinic acid in this cell line was demonstrated for the first time. In the light of these findings, it is shown that the cytotoxic impact of diffractaic and vulpinic acids varies according to different cell types and times.

Metastasis is associated with a range of molecular mechanisms, from cancer cells to loss of adhesion, invasion of stromal tissue, migration, penetration of blood vessels, disruption of the extracellular matrix, and spread to distant organs (Geho et al. 2005). Therefore, preventing the migration of cancer cells takes place among the essential subjects of anticancer studies. In the current work, a transwell migration assay was employed with the objective of assessing the impact of diffractaic and vulpinic acids on the migration ability of HeLa cells. The findings showed that both lichen metabolites could inhibit the migration of HeLa cells. It has been reported that diffractaic and vulpinic acids inhibit migration on human breast cancer MCF-7 and MDA-MB-453 cell lines, but their antimigratory effects in human cervical cancer were demonstrated for the first time (Kalın et al. 2022a, b).

Apoptosis can be induced through a variety of mechanisms, including alteration of the intracellular mitochondrial pathway (Galluzzi et al. 2018). In this research, the mRNA levels of both *BAX* and *BCL2* in HeLa cells treated with the $IC_{50}$ concentration of diffractaic acid were inconsistently increased by induction of apoptosis. However, an increase in the *BAX/BCL2* ratio indicates a proapoptotic state. The increasing *BAX/BCL2* ratio decreases cellular resistance to apoptotic stimuli and causes apoptosis (Ghavami et al. 2009). Furthermore, the increase in apoptotic cells (early and late) in the flow cytometry analysis results was in line with the qPCR results. In the literature, it has been reported that diffractaic acid has an apoptotic effect on breast cancer (Kalın et al. 2022a). When the apoptotic impact of vulpinic acid was evaluated in HeLa cells, the findings demonstrated that the mRNA level of *BAX* decreased, that of *BCL2* increased, and the *BAX/BCL2* ratio also decreased. It is known that the inhibition of apoptosis occurs in case of *BCL2* expression exceeding that of *BAX*. Interestingly, in contrast to the qPCR results, flow cytometry analysis showed that vulpinic acid significantly induced both apoptosis and necrosis in HeLa cells. In fact, it was observed that vulpinic acid increased the apoptotic rate of HeLa cells more than diffractaic acid. The current study focused on a single signaling pathway. Future research must investigate whether vulpinic acid is capable of inducing apoptosis through other pathways, including receptor-mediated or endoplasmic reticulum pathways. In the literature, the apoptosis feature of vulpinic acid on different cancer types, such as lung adenocarcinoma, pancreatic ductal adenocarcinoma, and hepatocellular carcinoma, has been reported (Kim et al. 2017). Similar to our results, another study found that vulpinic acid reduced the *BAX/BCL2* ratio in the breast cancer MCF-7 cell line (Kalın et al. 2022b), which could be explained by the different cancer cell lines and the variability depending on the dose and duration of the chemical substance.

The thioredoxin system regulates the intracellular redox balance by countering ROS and ultimately affects cell growth, differentiation, and death (Kondo et al. 2006). In addition, it has also been shown in the literature that thioredoxin system has extracellular functions (Bhatia et al. 2016). It has been reported that thioredoxin can be secreted by cells and bind to the external cell membrane,

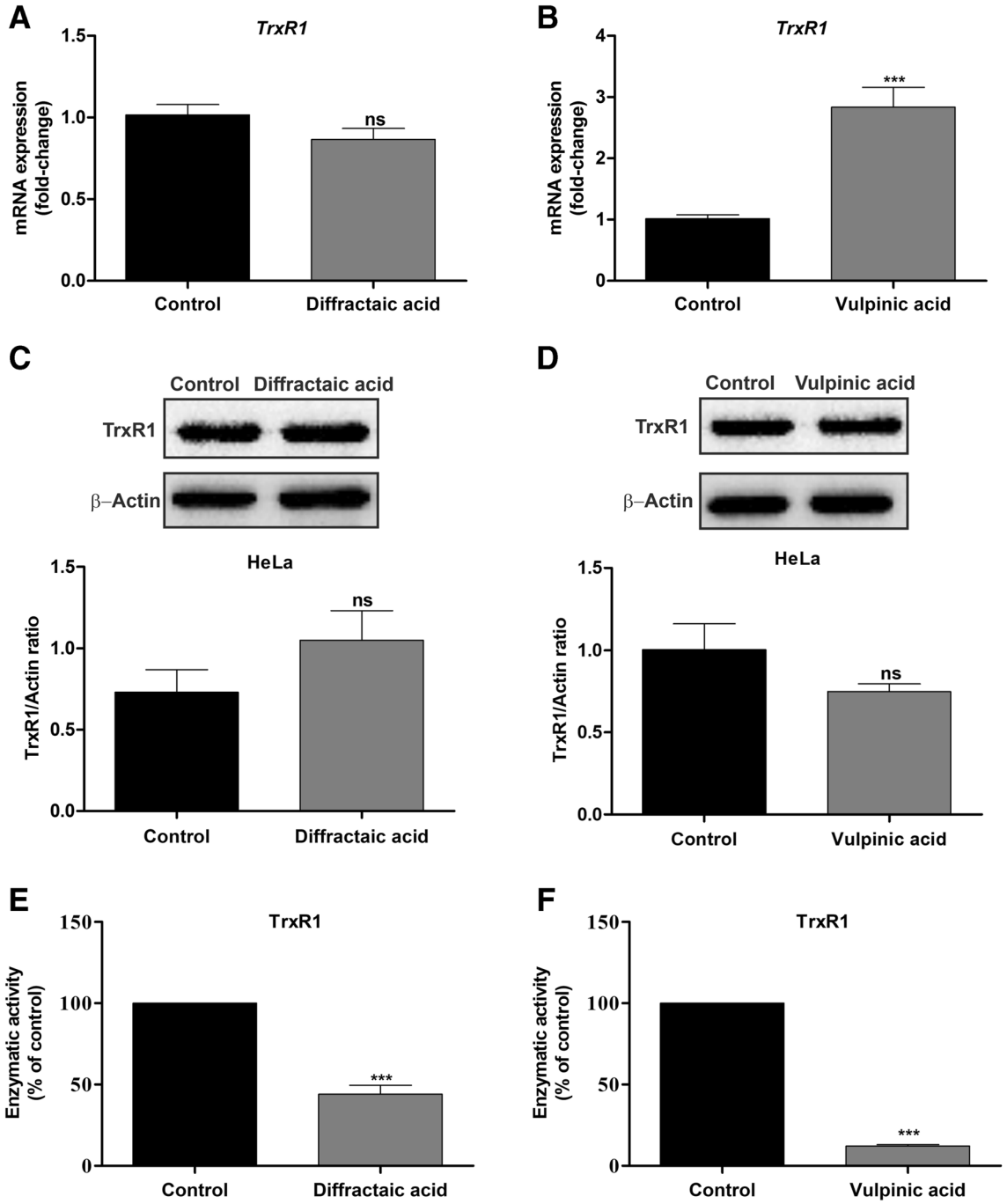



**Fig. 3** Inactivation of TrxR1 with diffractaic and vulpinic acids in HeLa cells. **A-B** The mRNA expressions of TrxR1 were evaluated via qPCR. **C-D** The TrxR1 protein expressions were determined by western blot and the results were compared with the control. β-Actin was used as an internal control. **E–F** TrxR1 enzyme activities were measured with/without treatment of each of these lichen metabolites in vitro. The experiment was conducted in three- biological and technical replicates. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ in relation to the control

where it can regulate cell-to-cell contact and contribute to redox regulation in the extracellular space (Rubartelli et al. 1992; Schenk et al. 1996; Banerjee 2012). As a result of this situation, it may also regulate cancer cell progression, proliferation, death and/or metastatic pathways through extracellular activity (Nakamura et al. 2006; Banerjee 2012). It has been reported that mitochondrial thiol-dependent redox systems including glutathione and thioredoxin may be altered after interaction with various anticancer agents (Witte et al. 2005).

It has been proven that TrxR1, as the thioredoxin system's component, is overexpressed and constitutively active in different cancer cells, thereby increasing the proliferation of cancer cells (Nguyen et al. 2006). Concerning the essential functions of TrxR1 in cancer development, TrxR1 is the primary target for anticancer drugs. Therefore, the interest in developing of new inhibitors of the this system as potential therapeutic agents has recently increased.

In the literature, diffractaic and vulpinic acids exerted more impacts in comparison with commercial anticancer drugs on inhibiting mitochondrial TrxR that was purified from rat lungs (Ozgencli et al. 2019). There are also several reports that inhibition of TrxR1 in different types of cancer can lead cells to apoptosis and suppress migration (Marzano et al. 2007; Bhatia et al. 2016; Kalın et al. 2022b, 2023; Günaydın et al. 2023). However, the absence of any data on TrxR1-related anticancer effects of both lichen metabolites on human cervical cancer highlights the importance of this study. In the present study, it was observed that diffractaic and vulpinic acids target TrxR1 in HeLa cells and cause its inhibition. Targeting TrxR1 with these lichen metabolites inhibits the physiological functions of TrxR1 and converts the enzyme to a NADPH oxidase for the purpose of producing superoxide anions, leading to the accumulation of ROS in the cells, thereby inducing oxidative stress and causing the death of cancer cells.

## Conclusions

The data acquired in the current work suggest that the inhibitory impact of diffractaic and vulpinic acids on the proliferation and migration of HeLa cells and their inducing effect on apoptosis may be related to TrxR1 inhibition. We believe that both lichen acids have the potential to be natural compounds for treating of cervical cancer.

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**Author Contributions** Conceived and designed the experiments: Dr. Büşra Budak (BB), Dr. Şeyda Nur Kalın (ŞNK), and Dr. Ömer Erkan Yapça (ÖEY). Performed the experiments: ŞNK and BB. Analyzed the data: ŞNK and BB. Contributed reagents/materials/analysis tools: ÖEY, ŞNK, and BB. Wrote the paper: ŞNK. All authors read and approved the manuscript's final version. The authors declare that all data were generated in-house and that no paper mill was used.

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**Data availability** The data supporting the findings of the present research are available on request from the corresponding author upon reasonable request.

## Declarations

**Competing ınterests** The authors declare no competing interests.

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