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ANTITUMORAL ACTIVITY OF DIFFRACTAIC ACID IN A375 HUMAN MELANOMA CELLS: INDUCTION OF APOPTOSIS AND INHIBITION OF CELL VIABILITY

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Abstract: Melanoma is one of the most aggressive forms of skin cancer due to its high metastatic potential and resistance to conventional therapies. Apoptosis resistance represents a key mechanism contributing to melanoma progression and therapeutic failure. Natural products have recently attracted increasing attention as potential sources of novel anticancer agents. Lichen-derived secondary metabolites, in particular, possess diverse biological activities, including cytotoxic and antiproliferative effects against various cancer types. Diffractaic acid, a depside compound isolated from several lichen species, has demonstrated anticancer potential in multiple tumor cell lines; however, its effects on malignant melanoma remain insufficiently explored. Therefore, this study aimed to investigate the cytotoxic and pro-apoptotic effects of diffractaic acid on A375 human melanoma cells. A375 human melanoma cells were cultured under standard conditions and treated with different concentrations of diffractaic acid (10–100 µg/mL). Cell viability was evaluated after 24 and 48 h using the MTT assay to determine the half-maximal inhibitory concentration (IC_{50}). Based on these results, three concentrations corresponding to ½×IC_{50}, IC_{50}, and 2×IC_{50} were selected for further analyses. Apoptotic activity was assessed after 48 h of treatment by measuring the levels of apoptosis-related proteins, including Bax, Bcl-2, caspase-3, caspase-8, caspase-9, cytochrome c, apoptotic protease activating factor-1 (Apaf-1), and p53 using ELISA-based assays. Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc test. Diffractaic acid significantly reduced the viability of A375 melanoma cells in a dose- and time-dependent manner. The IC_{50} values were determined as 59.5 µg/mL and 55.7 µg/mL after 24 and 48 h of treatment, respectively. Because stronger cytotoxic effects were observed at 48 h, this incubation period was selected for apoptosis analyses. Treatment with diffractaic acid at moderate and high concentrations significantly increased the levels of several pro-apoptotic markers, including caspase-3, caspase-9, cytochrome c, and Apaf-1, indicating activation of the mitochondrial apoptotic pathway. Additionally, caspase-8 levels increased at the highest concentration, suggesting involvement of the extrinsic apoptotic pathway. A significant elevation in p53 levels was also observed at the highest dose, indicating activation of p53-mediated apoptotic signaling. The findings demonstrate that diffractaic acid exerts significant cytotoxic and pro-apoptotic effects on A375 melanoma cells. The compound appears to induce apoptosis through activation of both intrinsic mitochondrial and extrinsic apoptotic pathways. These results suggest that diffractaic acid may represent a promising natural compound for further investigation as a potential therapeutic agent against malignant melanoma.

Keywords: Melanoma, A375 cell line, Lichen secondary metabolites, Diffractaic acid, Apoptosis, Cytotoxicity

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1. Introduction

Melanoma is the fifth most common cancer in adults and represents the most lethal form of skin cancer. It arises from the malignant transformation of melanocytes, the cells responsible for the synthesis of melanin, a photoprotective pigment. Although melanoma may develop in pigment-producing cells located in the eye, gastrointestinal tract, genital regions, paranasal sinuses, and meninges, it most frequently occurs in the skin as a consequence of ultraviolet radiation–induced damage. Clinically, melanoma is characterized by its highly aggressive nature, marked propensity for early metastasis, and poor prognosis in advanced stages (Saginala et al., 2021). Among solid tumors, melanoma exhibits one of the highest tendencies to metastasize to the brain, and brain metastases remain the leading cause of melanoma-related mortality. If left untreated, the disease is typically fatal due to widespread metastatic dissemination, although the time to progression varies depending on the histological and molecular subtype (Rabbie et al., 2019).

A hallmark of melanoma biology is its remarkable resistance to apoptosis. Melanoma cells often acquire alterations in pro- and anti-apoptotic signaling pathways, enabling them to evade programmed cell death even in the presence of cytotoxic stimuli. The molecular mechanisms underlying resistance to drug-induced apoptosis are complex and not yet fully elucidated (Wang et al., 2025). Therefore, a deeper understanding of

apoptotic regulation in malignant melanoma is essential for the development of more effective and durable therapeutic strategies. Although current treatment modalities—including surgical excision, sentinel lymph node biopsy, adjuvant therapy, chemotherapy, radiotherapy, immunotherapy, and targeted therapy—have improved patient outcomes, therapeutic resistance, disease recurrence, and systemic toxicity remain major clinical challenges. In particular, melanoma cells exhibit substantial resistance to conventional chemotherapeutic agents, underscoring the urgent need to identify novel compounds with potent antiproliferative and pro-apoptotic properties (Kavanagh et al., 2005; Davar et al., 2013).

In this context, natural products have historically served as a valuable source of bioactive molecules for drug discovery. Numerous anticancer agents currently used in clinical practice are derived from or inspired by natural compounds. Plants, fungi, and lichens constitute rich reservoirs of structurally diverse secondary metabolites with a broad spectrum of biological activities (Aldrich et al., 2022). Lichens, symbiotic associations between fungi and photosynthetic partners (algae or cyanobacteria), produce unique secondary metabolites that contribute to their survival under extreme environmental conditions. Secondary metabolites found in lichens include aliphatic, cycloaliphatic, aromatic, and terpenic compounds that exhibit significant biological and pharmacological effects, including anti-inflammatory, antiviral, antibacterial, analgesic, antipyretic, antiproliferative, and cytotoxic activities (Tripathi et al., 2022).

Recently, diffractaic acid, a lichen-derived secondary metabolite, has emerged as a compound of growing pharmacological interest. Diffractaic acid ($C_{20}H_{22}O_7$) is a depside derivative isolated from certain Usnea, Cladonia, and related lichen species (Ureña-Vacas et al., 2023). Accumulating evidence indicates that diffractaic acid possesses antimicrobial, analgesic, antipyretic, antioxidant, and gastroprotective properties (Ureña-Vacas et al., 2023; Sulukoğlu et al., 2024). Beyond these effects, several in vitro studies have reported its cytotoxic, antiproliferative, apoptosis-inducing activities in various cancer cell lines. Specifically, diffractaic acid has demonstrated notable pro-apoptotic and cytotoxic effects in breast cancer (MCF-7), cervical carcinoma (HeLa), colon carcinoma (HCT-116), glioblastoma multiforme (U87MG), hepatocellular carcinoma (HepG2), and lung cancer (A549) cells (Brisdelli et al., 2013; Emsen et al., 2018; Günaydın et al., 2023; Budak et al., 2024; Sulukoğlu et al., 2024). Despite these promising findings, the antitumoral potential of diffractaic acid in malignant melanoma, particularly with respect to cytotoxicity and apoptosis induction, has not been sufficiently investigated.

Cytotoxicity and apoptosis induction are fundamental parameters in the evaluation of anticancer agents, as effective therapeutic compounds are expected not only to inhibit cell proliferation but also to trigger regulated cell death pathways. The A375 human melanoma cell line, which harbors a BRAF mutation and closely reflects key molecular features of melanoma biology, is widely used as an in vitro model for assessing antitumor activity (Wang et al., 2022). Investigating the effects of diffractaic acid in this well-characterized model may provide valuable insights into its therapeutic potential against melanoma.

Therefore, the present study aims to evaluate the antitumoral activity of diffractaic acid on A375 human melanoma cells, with a particular focus on its cytotoxic and pro-apoptotic effects. Specifically, we sought to determine the impact of diffractaic acid on cell viability and to elucidate its role in the induction of apoptosis. We hypothesize that diffractaic acid decreases the viability of A375 melanoma cells in a dose-dependent manner and that this reduction is mediated, at least in part, through the activation of apoptotic pathways. By clarifying the cytotoxic and apoptosis-inducing properties of diffractaic acid in melanoma cells, this study aims to contribute to the growing body of literature on lichen-derived secondary metabolites as potential anticancer agents. The findings may support the consideration of diffractaic acid as a promising natural compound for further mechanistic investigations and preclinical evaluation, ultimately advancing the development of novel natural product–based therapeutic strategies for malignant melanoma.

2. Materials and Methods

2.1. Cell Lines and Culture Conditions

A375 cell line (human malignant melanoma cells) (Catolog Number: CRL-1619) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA. USA). A-375 melanoma cells were cultured in Dulbecco's minimal essentials medium (DMEM) with high glucose (Sigma, USA, Cat No. D6429), 10% fetal bovine serum (FBS), and 1% 10.000 units/mL of penicillin and 10.000 µg/mL of streptomycin. Cell culture flasks were kept in an incubator at the condition of 37 °C and 5% CO_2. In case of the cells being 80–90% confluent, they were sub-cultured to a fresh medium.

2.2. Preparation of Diffractaic Acid

Diffractaic acid (CAS Number: 436-32-8, $C_{20}H_{22}O_7$) was purchased from Cayman Chemical Company (MI, USA). A stock solution of diffractaic acid was prepared by dissolving it in dimethyl sulfoxide (DMSO) at a concentration of no more than 3%. This solution was then stored in a -20 °C freezer until it was needed. The serial dilution of diffractaic acid was performed with DMEM with the objective of obtaining the concentrations of 10, 25, 50, 75, and 100 µg/mL. The working solution was freshly prepared in the complete culture medium.

2.3. Evaluation of Cell Viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Mosmann, 1983). Cells were Mharvested from 25 cm^2 tissue culture flasks

upon reaching approximately 80% confluency and subsequently collected by centrifugation to obtain a cell pellet. The cells were resuspended and seeded into 96-well microtiter plates at a density of 5 × 10^5 cells/mL. Following seeding, cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 to allow cell attachment (Mosmann, 1983; Berridge et al., 2005; Stockert et al., 2012). Thereafter, adherent cells were treated with diffractaic acid at various concentrations (0, 10, 25, 50, 75, and 100 µg/mL). The stock solution of diffractic acid containing 3% DMSO was diluted with DMEM to obtain the desired working concentrations. This ensured that the final DMSO concentration in all treatment groups did not exceed 0.5% (v/v). To exclude any solvent-related effects, control cells were treated with DMEM containing an equivalent concentration of DMSO (0.5% v/v) under the same experimental conditions. This concentration of DMSO is widely reported to be subtoxic and does not significantly affect cell viability under standard *in vitro* conditions (Budak et al., 2024; Sulukoğlu et al., 2024). The plates were further incubated for 24 h and 48 h under standard culture conditions (37°C, 5% CO_2). At the end of each incubation period, the culture medium was removed, and 100 µL of fresh medium together with 20 µL of MTT solution (5 mg/mL, filter-sterilized; Sigma-Aldrich) was added to each well. Cells were then incubated for an additional 4 h at 37°C to allow formation of formazan crystals. Subsequently, the medium was carefully discarded and replaced with 150 µL DMSO to dissolve the formed formazan crystals. The plates were gently shaken at room temperature using an incubator shaker until complete solubilization was achieved. Absorbance values were measured at 540 nm using a microplate reader (VersaMax Microplate Reader). The percentage of cell growth inhibition was calculated using the following equation (equation 1) (Mosmann, 1983; Stockert et al., 2012):

$$Inhibition\ (\%) = (A_{control}-A_{sample})/A_{control} \times 100\% \quad (1)$$

The half-maximal inhibitory concentration (IC_{50}) values of diffractaic acid at both incubation periods were determined from dose–response curves generated by plotting percentage inhibition against compound concentration. Based on these results, ½×IC_{50}, IC_{50}, and 2×IC_{50} concentrations of diffractaic acid were calculated, and these doses were selected for use in subsequent experiments.

2.4. Assessment of Apoptosis

To elucidate the apoptotic effects of diffractaic acid on A375 melanoma cells, the levels of key apoptosis-related proteins, including Bax, Bcl-2, caspase-3, caspase-8, caspase-9, cytochrome c, apoptotic protease activating factor-1 (Apaf-1), and p53, were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's instructions (Lifespan Biosciences, Seattle, WA, USA). Briefly, A375 cells (5 × 10^5 cells per well) were seeded into appropriate culture plates and allowed to adhere overnight under standard incubation conditions (37°C, 5% CO_2). The cells were then treated with pre-determined concentrations of diffractaic acid for 48 h. At the end of the treatment period, culture supernatants were carefully collected and centrifuged at 1,500 × *g* for 10 min at 4°C to remove cellular debris, ensuring sample clarity and preventing interference with antigen detection. Subsequently, 200 µL of each clarified supernatant was added in duplicate to the ELISA plate wells pre-coated with capture antibodies specific to each target protein. Plates were incubated for 2 h at 37°C to allow antigen binding. Following incubation, wells were aspirated and washed twice with 400 µL of wash buffer to remove unbound substances, with care taken to minimize residual liquid and reduce background signal. Next, 200 µL of an enzyme-conjugated detection antibody solution, specific to the Bax, Bcl-2, caspase-3, caspase-8, caspase-9, cytochrome c, Apaf-1 and p53 proteins, was added to each well. The plate was then incubated for a further 2 h at 37°C. After this step, the wells were washed multiple times (typically 3–5 cycles) to ensure the removal of excess conjugate and to improve assay specificity. Following the washing procedure, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added to each well to initiate the enzymatic colorimetric reaction. The plates were incubated in the dark at room temperature for an appropriate development time, allowing for proportional color formation relative to the amount of bound analyte. The reaction was then terminated by adding stop solution, resulting in a color change from blue to yellow. The optical density values were measured at 450 nm, with wavelength correction at 570 nm to account for optical imperfections, using a microplate reader (VersaMax, Molecular Devices, California, USA). A standard calibration curve was generated for each analyte using known concentrations of recombinant protein standards provided in the kits. Protein concentrations in the samples were calculated by interpolation from the corresponding standard curves, ensuring that all sample readings fell within the linear dynamic range of the assay. All measurements were performed in at least duplicate, and the mean values were used for statistical analysis (Shankar et al., 2007; Fahim et al., 2025).

2.5. Statistical Analysis

Statistical analyses were performed using the (Statistical Package for the Social Sciences) SPSS software version 22.0 (SPSS Inc., Illinois, USA). All experiments were performed in triplicate and results were presented as mean ± standard deviation. For significant differences between control and diffractaic acid-treated groups, the p value between groups was determined by one-way analysis of variance (ANOVA) test followed by Tukey post-hoc analysis. $P<0.05$ was considered statistically significant (Genç and Soysal, 2018).

3. Results

3.1. Diffractaic Acid Inhibits Cell Viability on A375 Cells

A375 cells were treated with different concentrations of diffractaic acid (0-100 µg/mL) for 24 or 48 h. Administration of different concentrations of diffractaic acid caused a significant decline in cell viability for both incubation periods (P<0.05). For 24-hour incubation period, the cell viability for A375 cells was 99.8±1.1%, 91.3±1.9%, 79±2.8%, 53.6±2.6%, 32.1±1.7%, and 22.8±1.5% at diffractaic acid concentrations of 0, 10, 25, 50, 75, and 100 µg/mL compared to the control group, respectively. The IC_{50} value of diffractaic acid on A375 cells determined after a 24-hour incubation time was found as 59.5 µg/mL (Figure 1A). For 48-hour incubation period, the cell viability for A375 cells was 99.6±1.3%, 88.3±2.2%, 76.1±2.6%, 47.3±2.1%, 28.6±2.6%, and 21.2±1.7% at diffractaic acid concentrations of 0, 10, 25, 50, 75, and 100 µg/mL compared to the control group, respectively. The IC_{50} value of diffractaic acid on A375 cells determined after a 48-hour incubation time was found as 55.7 µg/mL (Figure 1B).

In terms of cytotoxicity, the 48-hour incubation period was more effective than the 24-hour period. Therefore, the 48-hour period was used as the basis for subsequent stages of the study. Diffractaic acid solutions at concentrations of 27.9 µg/mL (corresponding to ½×IC_{50}), 55.7 µg/mL (IC_{50}) and 111.4 µg/mL (2×IC_{50}), were applied to A375 cells. The cells were then incubated for 48 h and the levels of apoptosis-related proteins examined.

3.2. Diffractaic Acid Induces Apoptosis in A375 Cells

In this study, the effects of diffractaic acid on the apoptotic mechanism in the A375 melanoma cell line were investigated by treating the cells with three different concentrations of diffractaic acid (27.9 µg/mL, 55.7 µg/mL, and 111.4 µg/mL) followed by a 48-hour incubation period. After treatment, apoptosis-related molecular parameters were analyzed. The obtained findings demonstrated that diffractaic acid exerted concentration-dependent effects on apoptotic markers.

Analysis of Bax levels revealed that treatment with 27.9 µg/mL diffractaic acid did not cause a statistically significant change compared to the control group (p > 0.05). In contrast, treatments with 55.7 µg/mL and 111.4 µg/mL diffractaic acid significantly decreased Bax levels compared with the control group (P<0.01), indicating a suppressive effect on Bax level at moderate and high concentrations (Figure 2A).

Evaluation of Bcl-2 levels showed that 27.9 µg/mL diffractaic acid had no statistically significant effect on Bcl-2 level (P>0.05). However, treatment with 55.7 µg/mL and 111.4 µg/mL diffractaic acid significantly increased Bcl-2 levels compared to the control group (P<0.05), with a more pronounced increase observed at higher concentrations (Figure 2B). The Bax/Bcl-2 ratio, an important indicator of the balance between pro-apoptotic and anti-apoptotic signaling, remained unchanged following treatment with 27.9 µg/mL diffractaic acid (P>0.05). Conversely, treatments with 55.7 µg/mL and 111.4 µg/mL diffractaic acid resulted in a statistically significant decrease in the Bax/Bcl-2 ratio compared to the control group (P<0.01) (Figure 2C).

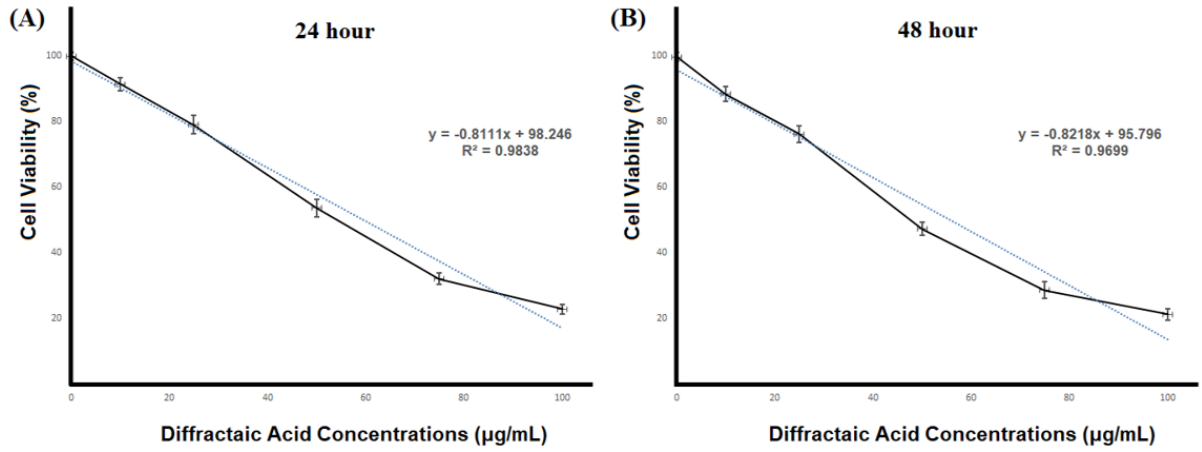

Figure 1. Effect of diffractaic acid on A375 cell viability. A375 cells were exposed to increasing concentrations of diffractaic acid (0–100 µg/mL) for (A) 24 h and (B) 48 h, and cell viability was assessed using an MTT-based colorimetric assay. Data are expressed as mean ± standard deviation (SD) of three independent experiments.

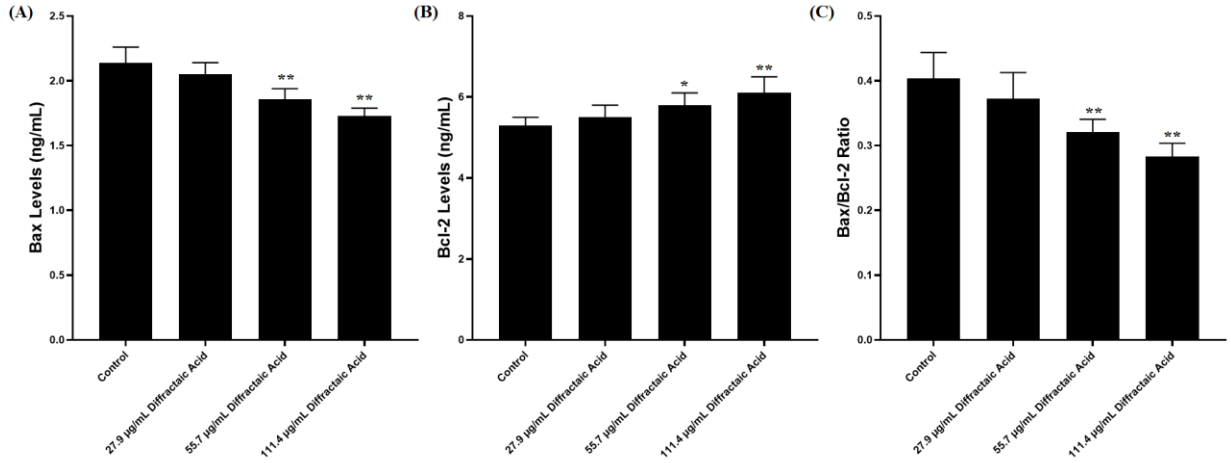

Figure 2. Effects of diffractaic acid on (A) Bax and (B) Bcl-2 levels and (C) Bax/Bcl-2 ration. Results are expressed as mean ± SD of three independent assays and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. (* P<0.05 *vs.* control group, ** P<0.01 *vs.* control group).

Regarding caspase-3 levels, which represent a key executioner caspase in apoptosis, no statistically significant alteration was observed following treatment with 27.9 µg/mL diffractaic acid (P>0.05). In contrast, both 55.7 µg/mL and 111.4 µg/mL treatments significantly increased caspase-3 levels compared with the control group (P<0.05) (Figure 3A). Evaluation of caspase-8, associated with the extrinsic apoptotic pathway, revealed that only treatment with 111.4 µg/mL diffractaic acid significantly increased caspase-8 levels compared to the control group (P<0.01). Treatments with 27.9 µg/mL and 55.7 µg/mL concentrations did not produce statistically significant changes (P>0.05) (Figure 3B). Similar findings were obtained for caspase-9, a key initiator caspase of the intrinsic apoptotic pathway. Treatment with 27.9 µg/mL diffractaic acid did not significantly affect caspase-9 levels (P>0.05), whereas 55.7 µg/mL and 111.4 µg/mL concentrations significantly increased caspase-9 level compared to the control group (P<0.01) (Figure 3C).

Assessment of cytochrome c levels, an important indicator of mitochondrial membrane permeabilization, demonstrated no significant change following treatment with 27.9 µg/mL diffractaic acid (P>0.05). However, both 55.7 µg/mL and 111.4 µg/mL treatments significantly increased cytochrome c levels compared with the control group (P<0.05) (Figure 4A).

Analysis of Apaf-1 level, a critical component of apoptosome formation, showed that 27.9 µg/mL diffractaic acid treatment had no significant effect (P>0.05). In contrast, treatments with 55.7 µg/mL and 111.4 µg/mL diffractaic acid significantly elevated Apaf-1 levels compared to the control group (P<0.05) (Figure 4B). Similarly, analysis of the tumor suppressor protein p53 demonstrated that a statistically significant increase was observed only following treatment with 111.4 µg/mL diffractaic acid (P<0.01), while 27.9 µg/mL and 55.7 µg/mL treatments showed no significant effect on p53 levels (P>0.05) (Figure 4C).

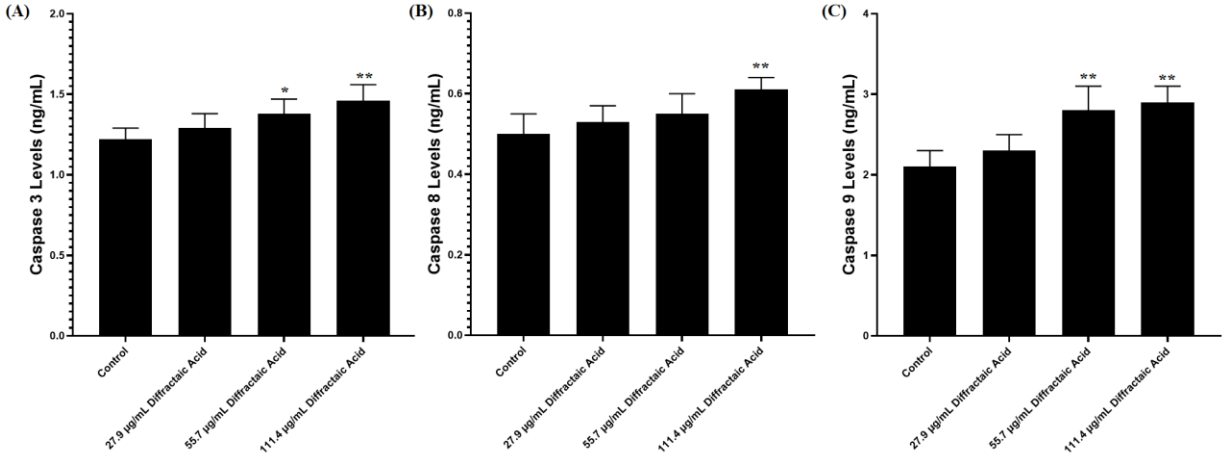

Figure 3. Effects of diffractaic acid on (A) caspase 3, (B) caspase 8 levels, and (C) caspase 9 levels. Results are expressed as mean ± SD of three independent assays and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. (* P<0.05 *vs.* control group, ** P<0.01 *vs.* control group).

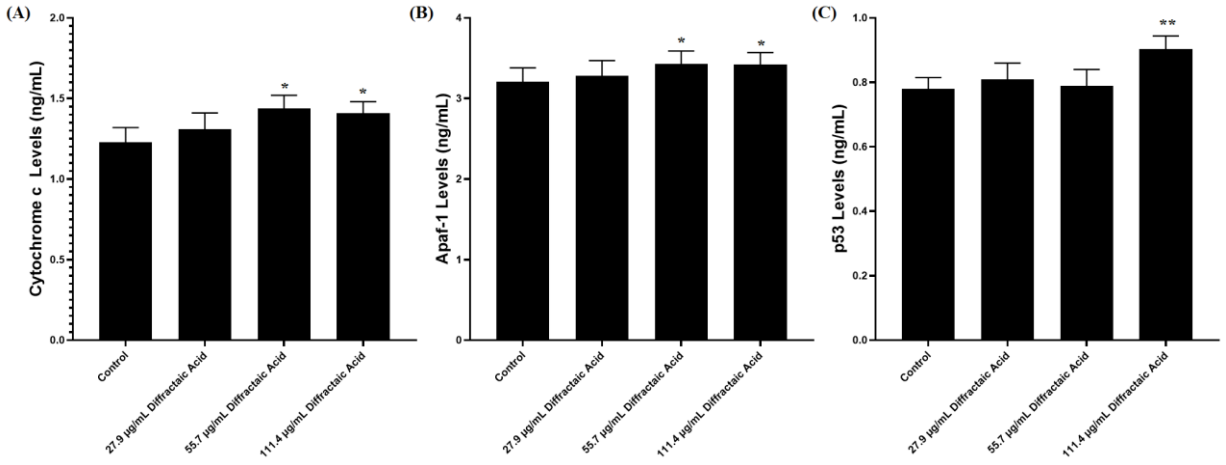

Figure 4. Effects of diffractaic acid on (A) cytochrome c, (B) Apaf-1, and (C) p53 levels. Results are expressed as mean ± SD of three independent assays and data were analyzed by one-way ANOVA followed by Tukey's multiple range post hoc test for multiple comparisons. (*P<0.05 *vs.* control group, ** P<0.01 *vs.* control group).

4. Discussion

Natural product–derived compounds have been used for centuries in the treatment of various diseases, including cancer, and many of these bioactive molecules have recently gained attention as complementary or alternative therapeutic agents (D'Incalci et al., 2005; Manach et al., 2009). Among natural sources, lichens represent an important reservoir of secondary metabolites with diverse biological activities. Traditionally, lichens have been used in folk medicine for the treatment of infections, dermatoses, tuberculosis, and respiratory diseases (Sepahvand et al., 2021). In recent years, increasing attention has been directed toward the potential anticancer properties of lichen-derived compounds due to their unique chemical structures and biological activities (Solárová et al., 2020). Several studies have demonstrated that lichen secondary metabolites exhibit antiproliferative effects against different cancer types, including gastric, hepatocellular, lung, breast, and prostate cancers (Yang et al., 2016; Eryılmaz et al., 2018; Kumar et al., 2020). Importantly, the cytotoxic activity of these compounds has been reported to vary depending on both the metabolite concentration and the cancer cell type.

In the present study, the antiproliferative activity of diffractaic acid was investigated in the human melanoma cell line A375. The MTT assay revealed that diffractaic acid exerted significant cytotoxic effects in a dose- and time-dependent manner, with an IC_{50} value of 55.7 µg/mL after 48 h of treatment. Several previous studies have reported the cytotoxic activity of diffractaic acid in different cancer cell lines. For instance, Kalın et al. (2022) reported IC_{50} values of 51.32 and 87.03 µg/mL in MCF-7 and MDA-MB-453 breast cancer cell lines, respectively, whereas Truong et al. (2014) observed IC_{50} values close to 90 µg/mL in MCF-7, HeLa and NCI-H460 cells. Similarly, Brisdelli et al. (2013) reported IC_{50} values of 93.4 µg/mL in MCF-7, 42.26 µg/mL in HCT-116, and 64.6 µg/mL in HeLa cells. More recent studies have identified IC_{50} values of 46.37 µg/mL in A549 lung cancer cells (Günaydın et al., 2023), 22.52 µg/mL in HeLa cells (Budak et al., 2023), and 78.07 µg/mL in hepatocellular carcinoma cells (Sulukoğlu et al., 2024). In contrast, diffractaic acid has been reported to exhibit minimal toxicity toward healthy human umbilical vein endothelial cells at concentrations up to 200 µg/mL (Kızıl and Ağar, 2017). These findings indicate that the cytotoxic activity of diffractaic acid is strongly cell-type dependent. Notably, the present study demonstrates for the first time the antiproliferative effect of diffractaic acid on A375 melanoma cells, suggesting that this compound may exhibit relatively strong activity in melanoma compared with several previously studied cancer cell types.

Melanoma is one of the most aggressive forms of skin cancer due to its high metastatic potential and resistance to conventional therapies. A major factor contributing to this resistance is the ability of melanoma cells to evade apoptosis. Therefore, reactivation of apoptotic signaling pathways has emerged as an important strategy in the development of novel anticancer agents. In this context, the present study investigated the molecular mechanisms underlying diffractaic acid–induced apoptosis in A375 melanoma cells by examining key components of both intrinsic and extrinsic apoptotic pathways (Do et al., 2021).

The intrinsic apoptotic pathway is primarily regulated by members of the Bcl-2 protein family, which control mitochondrial outer membrane permeability. Pro-apoptotic proteins such as Bax promote mitochondrial membrane permeabilization and facilitate cytochrome c release, whereas anti-apoptotic proteins such as Bcl-2 inhibit this process and support cell survival. Consequently, the balance between these proteins, commonly expressed as the Bax/Bcl-2 ratio, is considered a critical determinant of cellular susceptibility to apoptosis (Rossé et al., 1998). In the present study, treatment with diffractaic acid resulted in

a significant increase in Bcl-2 level accompanied by a decrease in Bax levels at higher concentrations, resulting in a reduction in the Bax/Bcl-2 ratio. These findings indicate a shift toward a pro-apoptotic intracellular environment and suggest that diffractaic acid may initiate mitochondrial dysfunction in melanoma cells (Eberle and Hossini, 2008).

Disruption of mitochondrial membrane integrity is known to trigger the release of cytochrome c into the cytosol, which subsequently interacts with apoptotic protease activating factor-1 (Apaf-1) to form the apoptosome complex. This complex activates initiator caspase-9, followed by activation of the executioner caspase-3, ultimately resulting in apoptotic cell death (Haraguchi et al., 2000). In agreement with this mechanism, the present study demonstrated increased levels of cytochrome c, Apaf-1, caspase-9, and caspase-3 following diffractaic acid treatment. These results collectively support the activation of the caspase-dependent mitochondrial apoptotic pathway in A375 melanoma cells.

In addition to mitochondrial signaling, apoptosis can also be initiated through the extrinsic pathway via death receptor activation, leading to the activation of caspase-8 (Fritsch et al., 2019). The observed increase in caspase-8 level at the highest concentration of diffractaic acid suggests that this compound may also stimulate receptor-mediated apoptotic signaling. Crosstalk between intrinsic and extrinsic pathways has been widely reported, and activation of caspase-8 may further amplify mitochondrial apoptosis through downstream caspase activation (Villunger et al., 2000).

Another important regulator of apoptosis is the tumor suppressor protein p53, which plays a central role in cellular responses to DNA damage and stress. Activation of p53 promotes apoptosis through modulation of Bcl-2 family proteins and enhancement of mitochondrial membrane permeability (Wei et al., 2021). The increase in p53 level observed following diffractaic acid treatment suggests that p53-mediated signaling may contribute to the initiation of apoptotic processes in melanoma cells. Taken together, these molecular alterations indicate that diffractaic acid induces apoptosis through coordinated activation of multiple apoptotic regulators.

Previous studies have also demonstrated that several lichen-derived metabolites induce apoptosis in A375 melanoma cells through similar molecular mechanisms. For instance, Cardile et al. (2017) reported that physodic acid promotes mitochondrial apoptosis by decreasing Bcl-2 level and increasing the Bax/Bcl-2 ratio. Yangın et al. (2022) showed that vulpinic acid stimulates apoptosis through activation of caspase-3, caspase-8, and Apaf-1. Other studies have reported that metabolites such as pannarin, sphaerophorin, atranorin, and usnic acid inhibit melanoma cell proliferation through activation of apoptotic pathways and modulation of caspase activity (Mohammadi et al., 2022; Büyük et al., 2024; Ensoy and Cansaran-Duman, 2026). These findings collectively suggest that lichen secondary metabolites commonly exert their antitumor effects by targeting mitochondrial integrity and activating caspase-dependent apoptotic signaling.

Despite these promising findings, several limitations should be acknowledged. First, the study was conducted exclusively in a single melanoma cell line, which may limit the generalizability of the results across different melanoma subtypes. Second, the experiments were performed in vitro, and thus do not fully reflect the complexity of in vivo tumor biology, including factors such as tumor microenvironment, bioavailability, and pharmacokinetics. Additionally, the study did not investigate the detailed upstream signaling pathways leading to apoptosis, nor did it assess potential off-target effects or long-term toxicity. The observed changes in apoptotic markers, while indicative, may also require further validation using additional functional assays.

5. Conclusion

Consistent with previous reports, the findings of the present study demonstrate that diffractaic acid induces apoptosis in A375 melanoma cells through modulation of key apoptotic regulators, including Bax, Bcl-2, cytochrome c, Apaf-1, and caspases. The coordinated activation of these molecules suggests that diffractaic acid promotes apoptotic cell death via interconnected intrinsic and extrinsic pathways. Although these results provide important insights into the antiproliferative and pro-apoptotic effects of diffractaic acid, certain limitations should be considered. Since the experiments were conducted in vitro, further in vivo studies are necessary to confirm these effects and to better understand the underlying molecular mechanisms. Future research should focus on validating these findings in different melanoma models and evaluating the pharmacokinetic properties, bioavailability, and safety profile of diffractaic acid. In addition, more detailed analyses of signaling pathways and potential combination therapies, as well as structural optimization of the compound, may help enhance its therapeutic potential.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	M.B.	S.K.
C	70	30
D	80	20
S	60	40
DCP	50	50
DAI	50	50
L	50	50
W	70	30
CR	70	30
SR	70	30
PM	80	20
FA	80	20

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

The necessary permission for the study was obtained from Van Yuzuncu Yil University Animal Experiments Ethics Commission (approval date: May 26, 2020, protocol code: 2022/05-03).

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