Original Article

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Molecular Basis of Apoptosis Induced by Taraxasterol on Human Melanoma Cell Line Growth Inhibition: An in-Vitro Study

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ABSTRACT

Background & Objective: Melanoma, one of the most lethal cancers, originates from epidermal layer. An advanced type of malignant melanoma represents a poor response to chemotherapy or other medications due to intrinsic and/or acquired resistance to antineoplastic drugs. Taraxasterol is a pentacyclic-triterpene agent mainly extracted from Dandelion herb with anti-proliferative and apoptotic features on cancer cells. Thus, this paper investigated the apoptosis pathway caused by Taraxasterol in the human melanoma cell line (hMCL).

Materials & Methods: hMCLs were treated with Taraxasterol and IC50 index was calculated using MTT assay. Then, apoptosis rate was evaluated by DNA Fragmentation Calorimetric technique. Finally, apoptosis pathway was investigated through various molecular laboratory assays.

Results: Low cellular viability level was found as concentration and time-dependent routes. Induction of apoptosis by IC50 value of Taraxasterol was found significantly ($p<0.05$) effective. Mitochondria membrane potential index was reduced by Taraxasterol significantly ($p<0.05$). Also, the cytosolic levels of cytochrome C and expression level of caspase 3, 8, and 9 genes in hMCL were increased significantly ($p<0.05$) following Taraxasterol administration.

Conclusion: Taraxasterol represents anti-proliferative and toxic effects against hMCL by induction of apoptosis.

Keywords: Apoptosis, Taraxasterol, Melanoma, Cell line, In vitro

Introduction

Melanoma, as a malignant type of dermal cancer arises from the melanocyte cells. Although this pathology is found in dermis, it is rarely detected in mouth, gut, or even eyes **(1)**. Melanoma is diagnosed with high invasion and rapid metastasis levels. The longevity of individuals with metastatic melanoma is approximately 6-10 months and <10% of cases can survive up to 5 years **(2)**. Etiologically, more than 65% of melanoma onset is associated with sunlight. Other risk factors are light skin, blond or red hair, large birthmarks, suppression of immune system, genetic polymorphisms, alcoholism, fleas, and multiple MACs, genetic factors, and family history **(3)**. There are many environmental factors inducing reactive oxygen species production and pathological reactions in dermis including smoking, microorganism invasion, or ultraviolet rays. Besides, antioxidants are biological or non-biological compounds with powerful inhibitory

effects against oxidation reactions. These compounds also diminish apoptosis and accelerate tissue health **(4)**. There is extensive scientific evidence regarding the application of herbal-based antioxidants in cancers with less toxicity. Several herbal extracts including Polyphenols, Flavonoids, Coumarins, Curcumins, and Terpenes contain antioxidant activity. Dandelion, or Taraxacum genus, is a member of the Asteraceae plant family with various applications in traditional medicine **(5)**. This plant is cultivated mainly in Bulgaria, Romania, Hungary, and Poland and in warm and temperate regions. In Russian, Indian, and Chinese traditional medicine, the Dandelion is widely known with beneficial liver-associated effects and anti-diabetic features **(6)**. Taroxasterol ((3β, 18α, 18α)-Urs-20(30)-en-3-ol) is extracted from various plants including Dandelion with definite anti-proliferation and apoptosis features on hepatocytes. Taraxasterol, as an important bioactive compound in Dandelion, is also known with anti-inflammatory and antioxidant activities **(7)**. According to the high drug resistance observed in melanoma cancer cells and the high annual death rate caused by melanoma cancer, this study was designed to assess apoptosis mechanism through the application of Taraxasterol on hMCLs.

Materials and Methods

Protocol of cell culture

A-375 cell line was cultured and incubated (37ºC, 5% CO2, and 95% humidity) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal calf serum (10%) and pen/strep antibiotic (1%) **(8)**.

Viability assay

To assess the viability index, 15×10^3 cells were cultured in a 96-well plate with 80% confluence (culture medium was changed daily). Then, various Taroxasterol concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µM) were added and incubated (24, 48, 72, and 96 hrs). The cellular solution was followed by adding 10µL of MTT and 100µL of DMSO. Finally, absorbance was read at 570nm. A well plate with no Taroxasterol was considered as the control group. Finally, the cell viability rate was calculated as; 100 × (absorption rate of control cells/absorption rate of treated cells). IC50 values were calculated using GraphPad Prism software (GraphPad Software Inc, v.5, San Diego, USA) **(9)**.

Toxicity assay

70×10^3 cells were cultures and incubated overnight in each well of 24-well plates. The culture medium (500µL) containing various Taroxasterol concentrations (from 0.78 to 100 µM) was added to the wells and followed by incubations for 24, 48, 72, and 96 hrs. Then 100µL of culture medium (from each sample) was transferred to another plate to measure Lactate Dehydrogenase enzyme activity (Cytotoxicity kit, Roche Chemical Co., Germany). Finally, the absorbance was measured at 490nm (based on a reference wavelength of 690 nm) **(10)**.

Apoptosis investigation

Following the cell treatment Taraxasterol, a suspension of cells (5×10^6 cells/ml) was prepared. 1ml of each sample was poured into a microtube (Microtube B of treated cells). The cells treated with Taroxasterol-free medium were used as control. Then the cells were centrifuged (200g, 10min, 4ºC) and the supernatant was transferred to a new microtube (Microtube S). 1ml of the solution was added to the sediment in microtube B. Triton X-100/Tris/EDTA was added and vigorously vortexed to release the fragmented chromatin after cell lysis. Then, to separate the fragmented chromatin from intact microtube B, the solution was centrifuged (20000g, 10min, 4ºC). The supernatant was transferred to microtube T and 1ml of X-100/Tris/EDTA solution was added to the sediment in microtube B. 1ml of Trichloroacetic acid solution 25% was added to all 3 microtubes and incubated overnight (at 4°C) for DNA precipitation. Then, all 3 microtubes were centrifuged (20000g, 10min, 4ºC). The supernatant was isolated and 160µL of Trichloroacetic Acid 5% was added to the remaining precipitation (heated at 90°C for 15min). 320µL of Phenylamine solution was added to each microtube and vortexed. The contents of the microtubes were incubated overnight (at room temperature). 200µL of each sample was used for measurement of absorbance at 600nm. DNA fragmentation (%) was calculated as; 100 × (absorption rate of S + absorption rate of T + absorption rate of B / absorption rate of S + absorption rate of T) **(11)**.

Mitochondrial membrane potential assay

Cell Meter™ JC-10 Mitochondrial Membrane Potential Assay kit (AAT Bioquest®, Inc, Sunnyvale, CA) was used to measure the effect of Taroxasterol on mitochondrial membrane potential. Following hMCL culture, the cells were treated with IC50 concentration of Taroxasterol. Then, X JC-10100 solution (50µL, part A) was added to 5ml of assay buffer A (part B). JC-10 dye solution (50µL) was added and incubated (37°C, 60min). Then, buffer B (50µL, part C) was added and fluorescence intensity of each well was measured (590/540 and 490/525 nm). The intensity ratio of fluorescence emission at wavelength of 590/525 was used for data analysis **(12)**.

Measurement of cytochrome C release from mitochondria

For this technique, a cytochrome C kit (Invitrogen Camarillo, CA) was used. Cells were cultured in flasks and treated with IC50 concentration of Taraxasterol. Flasks were washed (PBS buffer) and cells were separated by scraping method. 100×10^6 cells were washed twice (with PBS) and centrifuged (1500rpm, 5min). 1ml of cell extraction buffer was added to the sediment and incubated for 30min on an ice-pack, then

followed by vortex (10min intervals). The obtained extract was transferred to microcentrifuge tubes (13000rpm, 10min, 4ºC). Solutions with various concentrations (5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, and 0 ng/ml) were prepared from standard cytochrome C tubes. 100µL of standard dilution buffer was added to the Zero well. 100µL of standards or samples were added to wells and samples were diluted 1:10 in standard dilution buffer. The wells were covered with plates and incubated for 2hrs at room temperature. The contents of the wells were slowly aspirated, the solutions were removed, and the wells were washed 4 times with diluted washing buffer. Except for the blank well, 100µl of cytochrome C Biotin Conjugate solution was added. Then, the wells were covered by plate cover and incubated for 60min. The contents of each well were gently aspirated, drained, and discarded. The wells were washed 4 times with washing buffer. Except for the blank well, 100µl of diluted Streptavidin-HRP solution was added to each well. Then, the wells were covered and incubated for 30min at room temperature. The contents of the wells were gently aspirated, drained, and discarded, again. 100µl of Stabilized Chromogen solution was added. The plate was incubated for 30min in the dark at room temperature. 100µL of Stop Solution was added to each well and absorbance of each well was measured at 450 nm. The standard graph of absorption rate against the concentration of cytochrome C was prepared and the concentration of cytochrome C in all samples was calculated using standard graph **(13)**.

Gene expression assay

Gene expression of caspase-3 (F: *CAAACTTTTTCAGAGGGGATCG*, R: *GCATACTGTTTCAGCATGGCAC*) **(14)**, 8 (F: *GGATGGCCACTGTGAATAACTG*, R: *TCGAGGACATCGCTCTCTCA*) **(15)** and 9 (F: *TGTCCTACTCTACTTTCCCAGGTTTT*, R: *GTGAGCCCACTGCTCAAAGAT*) **(16)** were measured following hMCL exposure with IC50 concentration of taroxasterol. RNA was extracted (RNA isolation kit, DENAzist, Tehran, Iran) and cDNA was prepared (Vivantis Technologies, Selangor DE, Malaysia). GAPDH was also considered internal control (F: *TCCCTGAGCTGAACGGGAAG*, R: *GGAGGAGTGGGTGTCGCTGT*). Data analysis was applied by $Ct2^{-\Delta\Delta ct}$ technique. Real-time PCR was performed (SYBR Premix Ex Taq Technology, Takara Bio Inc., Shiga, Japan) based on the previously published protocol **(17)**.

Statistical data analysis

Normal destitution of data was assessed using Kolmogorov–Smirnov. One-way ANOVA Tukey post hoc test were hired for data analysis (using SPSS 16, SPSS Inc., Chicago, IL). Results were expressed as mean ± standard error, and $p < 0.05$ was considered significant **(18)**.

Results

Effect of Taroxasterol on hMCL Viability

The effects of different concentrations of Taroxasterol on cell viability index after 24, 48, 72, and 96 hrs are shown in Figure 1. The decreased survival rate was found significantly ($p<0.05$) after 24 and 48 hrs in concentrations of 3.12, 6.25, 12.5, 25, 50, and 100µM and after 72 and 96 hrs in all concentrations. Thus, decreased cellular viability was found dependent on concentration and time of drug exposure. The IC50 value calculated using MTT test was 183.3±9.23, 79.99±.85, 59.79±3.17, and 24.65±3.79µM respectively for 24, 48, 72, and 96 hrs **(Figure 1)**.

Cytotoxic effects of Taroxasterol on hMCL

Cytotoxicity of Taroxasterol on hMCL was found in dose and time-dependent manner. Toxicity levels were found significant ($p<0.05$) in concentrations of 6.25, 12.5, 25, 50, and 100µM respectively following 24 and 48 hrs and also 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µM respectively after 72 and 96 hrs **(Figure 2).**

Effects of Taroxasterol on hMCL apoptosis

The apoptosis induced by IC50 concentration of Taroxasterol on hMCL **(Figure 3)** was found significant ($p<0.05$).

Effects of Taroxasterol on mitochondrial membrane potential

The potential difference between both mitochondria membranes was detected significantly ($p<0.05$) with diminishing effects on hMCL following exposure to IC50 concentration of Taroxasterol **(Figure 4).**

Effects of Taroxasterol on cytochrome C release

The value of cytosolic cytochrome C was increased significantly ($p<0.05$) following exposure of IC50 concentration of Taraxasterol on hMCL **(Figure 5)**.

Effects of Taroxasterol on caspase-associated genes expression

Following the exposure of IC50 concentration of Taraxasterol on hMCL, accelerated levels of caspases 3, 8, and 9 gene expression were detected in hMCL significantly ($p<0.05$) **(Figure 6).**

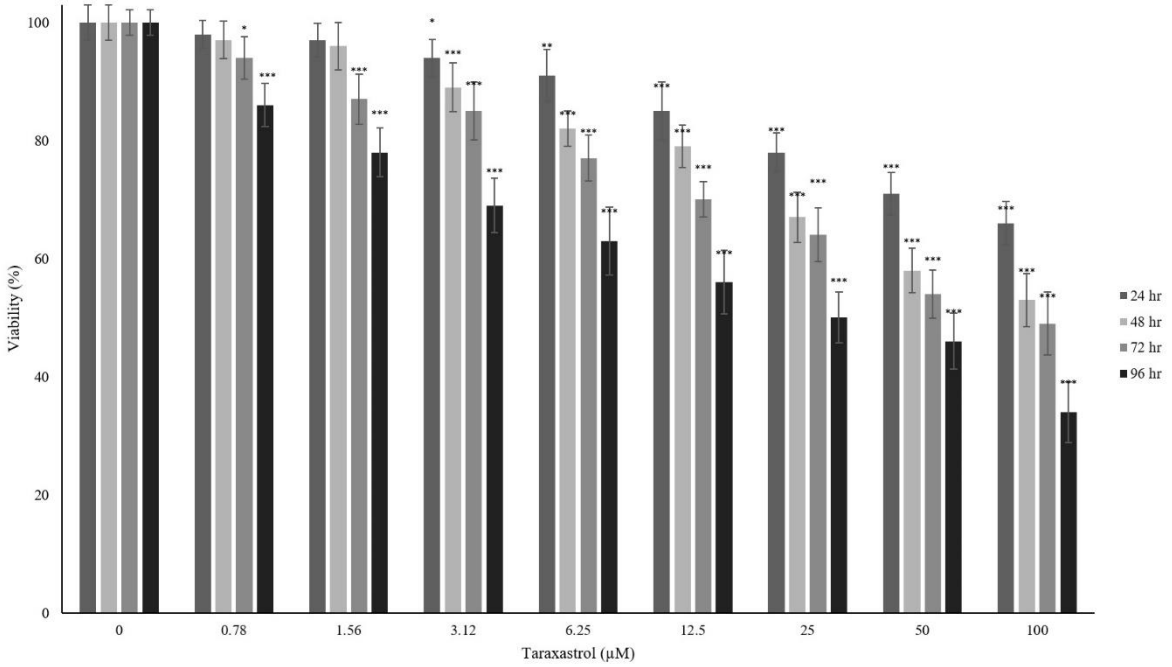

Figure 1. The effect of Taroxasterol on the viability of hMCL following 24, 48, 72, and 96 hours of treatment with different concentrations (evaluated by MTT assay). The control group received the same volume of medium without drugs. * indicates p<0.05, ** indicates p<0.01 and * indicates p<0.001 compared to the control.**

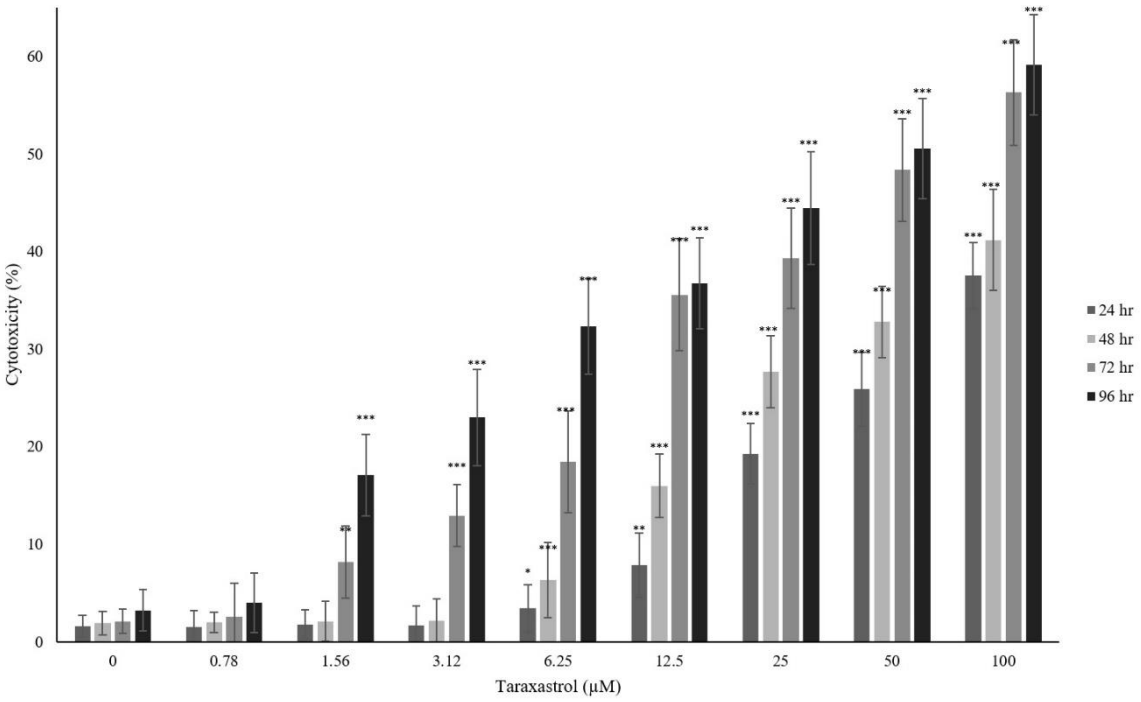

Figure 2. Taroxasterol toxicity on hMCL following 24, 48, 72, and 96 hours of treatment with different concentrations (evaluated by Lactate Dehydrogenase assay). The control group received the same volume of medium without drugs. * indicates p<0.05, ** indicates p<0.01 and * indicates p<0.001 compared to the control.**

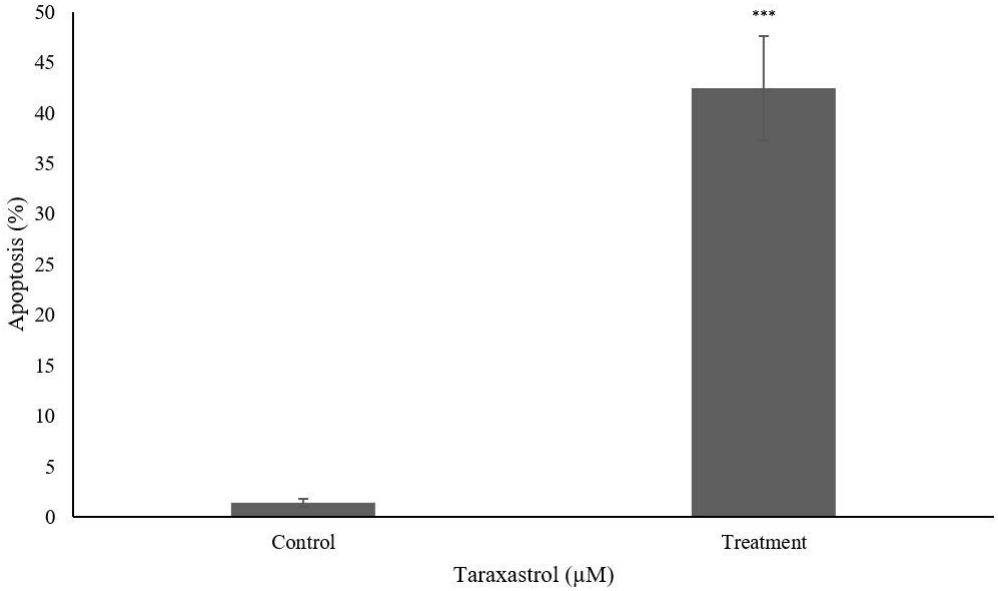

Figure 3. Apoptosis rate induced by Taraxasterol on hMCL following 24 hours of treatment with IC50 concentration (evaluated by Phenyldiamine assay). The control group received the same volume of medium without drugs. * indicates p<0.001 compared to the control.**

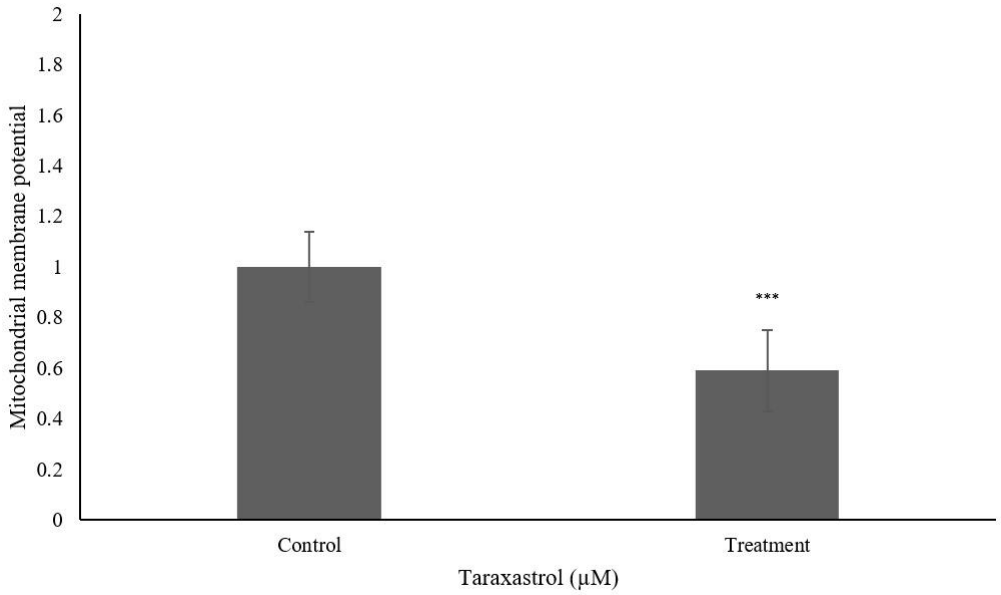

Figure 4. Potential difference between both mitochondrial membranes in hMCL following 24 hours of treatment with IC50 concentration. The control group received the same volume of medium without drugs. * indicates p<0.001 compared to the control.**

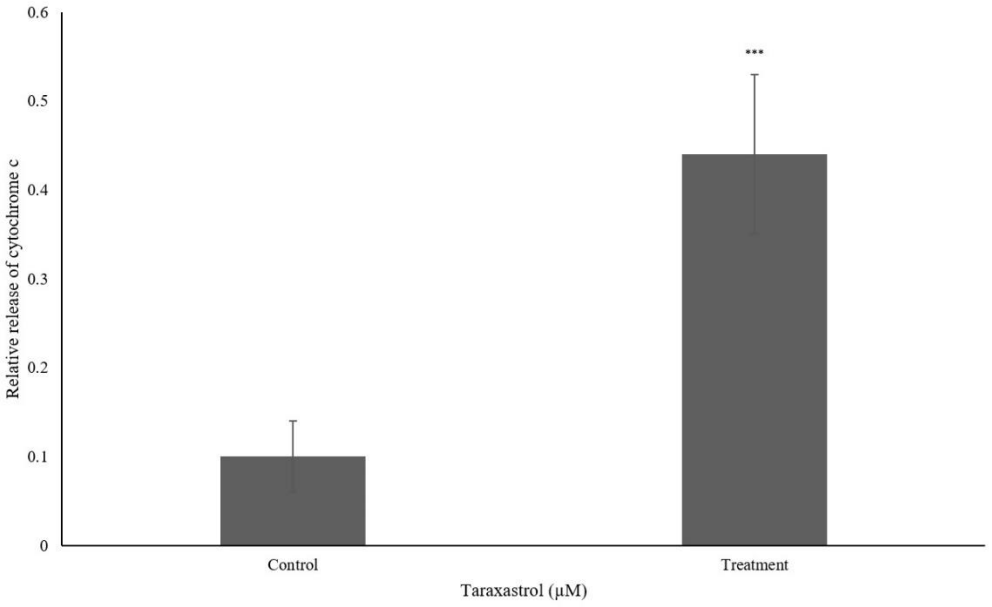

Figure 5. The levels of cytosolic Cytochrome C in hMCL following 24 hours of treatment with IC50 concentration of taroxasterol. The control group received the same volume of medium without drugs. * indicates p<0.001 compared to the control.**

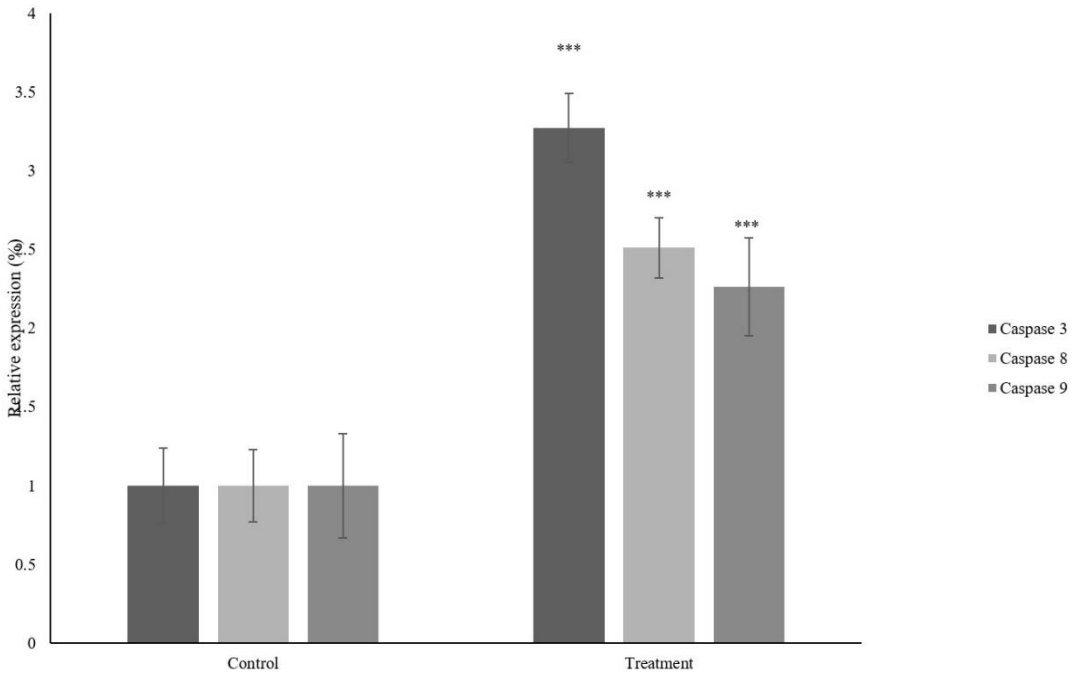

Figure 6. The gene expression of Caspase 3, 8, and 9 in hMCL following 24 hours of treatment with IC50 concentration of taroxasterol. The control group received the same volume of medium without drugs. * indicates p<0.001 compared to the control.**

Discussion

In the preset in-vitro study, the effects of Taroxasterol on hMCLs were investigated and the molecular changes were assessed including survival rate, mitochondrial membrane integrity, apoptosis, mitochondrial membrane potential, and expression of Caspase genes. The results of this study showed that Taroxasterol can induce apoptosis in melanocyte cell lines depending on the concentration and time routes of the drug. Previous studies approved the anti-cancer effects of the Dandelion plant. In 1981, for the first time, the extract of Dandelion represented antitumor activity **(19)**. According to the studies representing the Dandelion with anti-cancer features on malignant cells, this agent contains cytotoxicity on cancer cells through upregulation of TNF-α and IL-1α **(20)**. TNF-α and IL-1α are pro-inflammatory cytokines and act synergistically in various cellular processes **(21)**. In addition, the activation of MMPs (type 2 and 9) induces critical cellular roles including degradation of extracellular matrix components and modulation of inflammatory responses **(22)**. MMP types 2 and 9 have implications in various pathological processes and degradation of collagen I and IV. They also can affect the activity of interleukins and chemokines. Overexpression of MMP-2 and MMP-9 has been correlated with the progression of many tumor types, and high levels of MMP-9 are associated with various chronic inflammatory conditions **(23)**. MMPs (types of 2 and 9) promote tumor growth, invasion, and metastasis by degradation of extracellular matrix and modulation of immune responses. The activation of MMP-2 and MMP-9 can lead to the degradation of tumor suppressor proteins and the promotion of angiogenesis as two main key factors in tumor progression **(24)**. Stem cells initiate tumorigenesis and conventional cancer treatments often exhibit incomplete and transient therapeutic effects, resulting in tumor regression followed by recurrence. This phenomenon can be attributed to the presence of multiple resistance mechanisms within the tumor microenvironment. Sox2 dysregulation is associated with the maintenance of cancer stem cell characteristics, which contribute to tumorigenesis and therapeutic resistance in various types of cancers. Specifically, Sox2 plays a pivotal role in regulation of self-renewal and differentiation potential of cancer stem cells leading to tumor growth, metastasis, and response to treatment **(25)**. The overexpression of Sox2 has been correlated with the initiation and progression of tumors, as well as development of resistance to therapeutic interventions. Furthermore, Sox2 is implicated in regulatory networks which are critical for cellular processes such as proliferation, apoptosis, and metastasis. Thus, Sox2 is a promising candidate for

targeted cancer therapies. Abnormal expression of Sox2 in cancer cells plays a critical role in preservation of cancer stem cell characteristics, which are associated with tumor recurrence and resistance to conventional cancer therapies. This assertion highlights the importance of Sox2 in tumor biology, as its dysregulation can lead to persistence of self-renewal capabilities and contribute to tumorigenesis. Research indicates that increased levels of Sox2 are linked to several cellular processes of progression, including enhanced proliferation, clonogenicity, and tumorigenicity. Moreover, Sox2 overexpression is associated with various mechanisms of resistance to standard anticancer treatments, encompassing increased invasion, migration, and stem-like properties, as well as chemoresistance. A deep understanding of the role of Sox2 in tumorigenesis is an important aspect of cancer research. Following the discovery of the molecular pathways of Sox2, novel treatment strategies can be designed **(26)**. Inhibition of Sox2 expression diminishes the malignant characteristics associated with breast cancer, particularly in terms of invasion and migration. Furthermore, Sox2 expression exhibits a significant correlation with TNM staging and lymph node metastasis in patients with breast cancer. Consequently, Sox2 is regarded as a potential therapeutic target in breast cancer due to its involvement in promoting tumorigenicity and contributing to therapy resistance. The selective mechanism of TRAIL is a valuable candidate for therapeutic strategies aimed at enhancing cancer treatment efficacy while minimizing damage to normal tissues **(27)**. Despite its role as an anticancer, many cancer cells exhibit resistance to TRAIL-induced apoptosis, which significantly limits its therapeutic efficacy. Thus, TRAIL resistance is a crucial aspect in advancement of TRAIL-based cancer therapies **(28)**. TRAIL exerts its pro-apoptotic effects by binding to its cognate receptors, DR4 and DR5, which are expressed on surface of cancer cells. This ligand-receptor interaction initiates a signaling cascade which leads to activation of caspases, a family of enzymes responsible for cellular proteins cleavage. The findings of this study indicated that Taroxasterol exhibits concentration- and time-dependent cytotoxicity in melanoma cells. Lactate dehydrogenase is a cytosolic oxidoreductase enzyme which catalyzes the conversion of pyruvate to lactate under conditions of hypoxia or anoxia, while concurrently facilitating the conversion of NADH to NAD+. The integrity of cell membrane is compromised in response to cytotoxic agents, resulting in the release of lactate dehydrogenase (LDH) into the culture medium. Consequently, the activity of LDH in culture medium serves as an indicator of cell membrane integrity. Diaphorase utilizes NADH and H+ to reduce tetrazolium salt **(29)**. This process is commonly used in cell-based assays to measure NADH and NAD+ levels, and it is an important tool for assessment of cellular redox reactions and metabolic activity **(30).** Thus, the activity level of lactate dehydrogenase in culture medium can serve as a reliable indicator of cell membrane integrity. These results further indicated the potential role of Taraxasterol on apoptosis induction in melanoma cells through DNA fragmentation. Following the restriction enzyme reaction, the resulting products were separated by gel electrophoresis, and desired DNA fragment was subsequently purified for use in subsequent experiments. Following the restriction enzyme reaction, 180 to 200 restriction fragments were generated. The purified restriction fragment was subsequently utilized in the subsequent experiments. The results indicated that induction of apoptosis in melanoma cells is associated with a decrease in mitochondrial membrane potential differences. Mitochondria, in addition to serving as the primary source of ATP production under aerobic conditions and regulating the energy levels necessary for cellular survival, can also generate signals that initiate apoptotic pathway **(31)**. Mitochondrial alterations during apoptosis encompass the production and release of ATP, as well as opening of permeability transition pores. Opening of these pores leads to the loss of mitochondrial membrane potential, an increase in matrix volume, and subsequent disruption of inner membrane structure. Totally, apoptotic factors can be released within the cytoplasm. This study offers valuable insights into the influence of Taraxasterol into intracellular environment. Cytochrome C is bound to cardiolipin in inner mitochondrial membrane through electrostatic and hydrophobic interactions **(32)**. During apoptosis, high levels of ROS are produced in mitochondria, and cardiolipin is oxidized. Cytochrome C is released from the associate complex through outer membrane pores into cytosol. This study elucidates the role of cytochrome C in apoptotic pathway and its interaction with cardiolipin. In this project, to elucidate the apoptosis pathway activated by Taroxasterol, the activity and expression levels of caspases 3, 8, and 9 in melanoma cells were investigated. Results demonstrated that treatment with Taroxasterol activates caspases 8 and 9 **(33)**. Increased activity of caspase 9 and 8 is associated with increased activity of caspase 3. These results suggest that Taroxasterol induces apoptosis through the internal pathway dependent on mitochondria and external pathway independent of mitochondria.

Conclusion

Taraxasterol by various molecular mechanisms can potentially induce a low rate of cell viability and high levels of apoptosis. This anti-cancer drug induces cytotoxic effects through a decrease in mitochondrial membrane potential and acceleration in cytosolic Cytochrome C along with upregulation of gene expression of caspases 3, 8, and 9. It is strictly recommended to investigate the potential anti-proliferative features of Taraxasterol on animals infected by melanocytoma malignant tumors.

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Authors' Contribution

Conceptualization, F.K-H. and C.J.; methodology, M.P.; software, I.R.; validation, A.A.; writing—original draft preparation, S.H.; editing, C.J.; supervision, F.K.; project administration, F.K.; funding acquisition, C.J".

Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethics Approval and consent to participate

This research project was approved by Kermanshah University of Medical Sciences (IR.KUMS.REC.1400.094).

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