https://doi.org/10.31925/farmacia.2022.5.5 **ORIGINAL ARTICLE**

POLYPHENOLS CONTENT AND *IN VITRO* ANTITUMOUR ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *VISCUM ALBUM* IN TWO PIGMENTED AND UNPIGMENTED SKIN CANCER CELL LINES

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Abstract

Malignant melanoma is the most aggressive type of skin cancer, with an increasing annual incidence. In addition to cutaneous melanoma, another form of melanoma with an increased death rate is uveal melanoma. With both types of melanomas, there is a risk that the malignant cells will become resistant to conventional antitumour therapy. Thus, the present study aimed to evaluate the antitumour effect of a hydroalcoholic extract of *Viscum album* (VAex) on a pigmented cell line (B164A15) as well as unpigmented (A431). The cytotoxic effect was also followed in the case of a healthy human keratinocyte cell line. The results showed that at low concentrations (< 500 μg/mL), VAex has a selective cytotoxic effect, with healthy cells not being affected. In addition, in the case of tumour cells, VAex exerted a concentration-dependent cytotoxic effect, causing morphological and structural changes in the nucleus, with an apoptotic-like effect. The most affected cells were the unpigmented ones (A431), requiring additional studies to elucidate the mechanism of antitumour action, but also to determine the influence of melanin on the biological action of VAex.

Rezumat

Melanomul malign este cel mai agresiv tip de cancer de piele, cu o incidență anuală în creștere. Pe lângă melanomul cutanat, o altă formă de melanom cu o rată crescută a mortalității este melanomul uveal. În cazul ambelor tipuri de melanoame, există riscul ca celulele maligne să devină rezistente la terapia antitumorală convențională. Astfel, studiul de față a avut ca scop evaluarea efectului antitumoral al unui extract hidroalcoolic de *Viscum album* (VAex) asupra unei linii celulare pigmentate (B164A15), precum și asupra unei linii celulare nepigmentate (A431). Efectul citotoxic a fost urmărit, de asemenea, în cazul unei linii celulare de keratinocite umane sănătoase. Rezultatele au arătat că, la concentrații mici (< 500 μg/mL), VAex are un efect citotoxic selectiv, celulele sănătoase nefiind afectate. În plus, în cazul celulelor tumorale, VAex a exercitat un efect citotoxic dependent de concentrație, provocând modificări morfologice și structurale în nucleu, cu un efect de tip apoptotic. Cele mai afectate au fost celulele nepigmentate (A431), fiind necesare studii suplimentare pentru elucidarea mecanismului de acțiune antitumorală, dar și pentru a determina influența melaninei asupra acțiunii biologice a VAex.

Keywords: *Viscum album*, hydroalcoholic extract, polyphenols, melanoma, viability

Introduction

In terms of global health concerns, cancer remains one of the most important problems. Accordingly, malignancy ranks as the leading cause of death world-wide, and the number of cancer deaths is on the rise [1]. Consequently, cancer has emerged as a hot topic for researchers. While there has been substantial progress in the field, cancer continues to pose a global health threat. From a pathophysiological standpoint, cancer has a number of malignant features, including an increased number of cells proliferating and invaded neighbouring tissues, thereby leading to metastases [2].

Malignant melanoma has been one of the most widely studied cancers in recent years. Throughout the past few years, the incidence of cutaneous melanomas has significantly increased. By 2020, this type of cancer accounted for nearly 2% of all cancer cases and 0.6% of cancer deaths [3]. After cutaneous melanoma, ocular melanoma is the second most common form of melanoma. Among the types of cancer that affect the eyes, uveal melanoma occupies the first position. Due to its high mortality rate, uveal melanoma remains an important topic despite its relatively low incidence [4]. The main pathophysiological mechanism considered to be the promoter of ocular melanoma consists in the oxidative alterations of the pigmented tissues [5].

Another negative side of melanoma is the refractory mode of response to classic treatment, including chemotherapy and radiation therapy [3]. In addition, conventional treatments such as chemotherapy, radiation therapy or surgery have a wide range of side effects with a major impact on patients' quality of life [6]. For this reason, in recent years the focus of researchers has been on the plant kingdom as a complementary source of active anti-tumour compounds. In recent decades, research has provided evidence of the antitumour effects of phytocompounds. Currently, there are a remarkable number of compounds of natural origin, either used alone or in combination with conventional antitumour drugs, in preclinical and clinical studies [7]. Moreover, between 1983 and 1984, more than 60% of FDA-approved antitumour drugs for human use were of natural origin [8].

Viscum album L., popularly known as mistletoe, is a semi-parasitic plant that grows on various host trees and is part of the *Santalaceae* family. Mistletoe has been used as a complementary anti-tumour therapy especially in Central Europe. The main mechanisms of antitumour action already documented in the literature include the immunomodulatory, cytotoxic and pro-apoptotic actions of mistletoe [9]. Research in the field has sought to make a correlation between the anti-tumour action and the chemical composition of mistletoe extracts. Due to its rich composition of flavonoids, phenolic compounds, triterpenes, oligo and polysaccharides and many others, the mechanism of antitumour action remains incompletely elucidated [10].

Based on these premises, the first purpose of this article was to evaluate the composition of the hydroalcoholic extract of *Viscum album* (VAex). In addition, cytotoxic action was determined using two skin cancer cell lines: skin epidermoid carcinoma – A431, as well as a murine melanoma tumour cell line – B164A5 and, also a healthy human keratinocyte cell line. Finally, by using the staining technique, the effect exerted by VAex on the structure of the nucleus was highlighted, thus providing an image of the type of cell death induced by the extract.

Materials and Methods

Reagents

Standards of polyphenols, solvents (ethanol, methanol acetonitrile) were purchased from Sigma Aldrich (Merck KGaA Darmstadt, Germany).

The cell lines, A431 - skin squamous cell carcinoma (ATCC® CRL-1619TM), purchased from ATCC (American Type Cell Collection), B164A5 - murine melanoma (94042254; ECACC), purchased from ECACC (European Collection of Authenticated Cell Cultures) and HaCaT – keratinocytes purchased from CLS Cell Lines Service GmbH, Germany, were cultured in the culture-specific medium represented by Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30-2002™), completed with 10% foetal bovine serum (FBS, Gibco, USA) and 1% of antibiotic mixture (penicillin and streptomycin, Sigma Aldrich, Merck KGaA Darmstadt, Germany).

The antibiotic mixture penicillin and streptomycin, phosphate-buffered saline (PBS), trypsin EDTA, Trypan blue and Alamar blue (resazurin) were acquired from Sigma Aldrich, Merck KGaA, Germany. Culture medium, Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30-2002™) was purchased from ATCC and foetal bovine serum (FBS, Gibco) was purchased from Thermo Fisher Scientific, Inc., Waltham, MA, USA. All the reagents were of analytical standard purity and were applied according to the manufacturers' recommendations.

Preparation of extract

Viscum album young leaves utilized in current research were harvested from Timiș County (Western Romania) and certified at the Pharmaceutical Botany Department (VS/S2/2019 no. of voucher herbarium specimen), Faculty of Pharmacy, "Victor Babeș" University of Medicine

and Pharmacy, Timișoara, Romania. The plant material was dried at room temperature and stored in optimal conditions until extraction stage. To obtain the *Viscum album* total extract, the maceration method was chosen. In short, the following stage were realized: (i) maceration of 10 g of crushed and homogenized dried leaves in 50 mL of ethanol (50%) for five days; (ii) filtration of extraction mixture; (iii) solvent removal by using a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator package, Germany) under vacuum; and (iv) lyophilisation and storage at 2 - 8°C until further evaluations.

Liquid chromatography-mass spectrometry analysis

LC-MS (liquid chromatography coupled with mass spectrometry) analysis was realized on Agilent analytical system (6120 from Santa Clara, CA, USA) equipped with: HPLC system - 1260 Infinity HPLC with degasser, quaternary pump, column thermostat, detector and manual injector; quadrupolar mass spectrometer with electrospray ionization source (ESI); and computer with specific software, OpenLAB CDS ChemStation Workstation, for data processing.

Analysis details were reverse phase column (Zorbax Eclipse Plus C18; 3.0 × 100 mm × 3.5 µ); gradient program; mobile phase A 0.1% acetic acid in water and mobile phase B methanol; gradient linear elution (0 - 5 min. 5% mobile phase B in linear elution, 5 - 35 min. gradient elution reaching 90% mobile phase B, proportion kept until 38 min. and ending with 5% mobile phase B until 40 min); flow rate 1 mL/min; injection volume 10 µL; column temperature 40°C; UV detection at 280 and 320 nm; negative ion mode; ESI in the single ion monitoring (SIM) mode; capillary voltage 3500 V; dry gas flow 12 L/min at 350°C; nebulizer pressure 55 psig. The external standard method (0.05 - 2.5 µg/mL range) was used for calibration curves to quantify the individual polyphenols, to establish the *m/z* scale of the mass spectrum, an external calibration standard ESI Tuning Mix was employed [12].

Cell culture

Three cell lines were used in the present study, namely: A431 – skin epidermoid carcinoma (ATCC® CRL-1619™), B164A5 (94042254; ECACC) – murine melanoma and HaCaT – keratinocytes from CLS Cell Lines Service GmbH (Eppelheim, Germany). The cells were cultured in culture-specific medium, Dulbecco's Modified Eagle's Mediumm high glucose in which were added 10% foetal bovine serum (FBS, Thermo Fisher Scientific, Inc., Waltham, MA, USA). A 1% penicillin/streptomycin solution (Pen/Strep 10,000 U/mL; Sigma Aldrich, Germany) was used to prevent microbial contamination. Throughout the experiment, the cells were maintained at constant temperature (37°C) and 5% CO_2. Countess™ II Automated Cell Counter was used to determine the number of cells, and the count was performed in the presence of Trypan blue [13].

Cell viability assessment

To determine the effect on cell viability of *Viscum album* extract, the Alamar Blue method was applied. For this purpose, the cells were cultured in 96-well plates in a 1 x 10^4 cell/well number. After reaching a confluence of approximately 90%, the cells were stimulated for 24 hours with five concentrations of VAex (50, 100, 250, 500 and 1000 µg/mL). At the end of the 24-hour period, the Alamar Blue method described above was applied [11]. Thus, a volume of 10 µL/well of Alamar Blue was added, and the cells were incubated for 3 hours. After this time, the absorbance spectra were measured at 570 nm and 600 nm using the xMark™ Microplate spectrophotometer (Bio-Rad). The results were expressed as a percentage of viable cells (%) calculated by applying the formula described in the literature [14].

Nuclear staining

The identification of the type of cell death that occurred as a result of cell stimulation with VAex was performed by a staining test with Hoechst 33343. For this purpose, the cells were cultured in 12-well plates in a number of 1 x 10^5 cells/well number. After reaching a suitable confluence of 80 - 90%, the cells were stimulated with two concentrations of VAex (50 and 1000 µg/mL) for 24 hours. For data analysis, a 5 µM solution of Staurosporin was used as a positive control for the induction of apoptosis. Cells were stimulated with Staurosporin for 3 hours. After this time, the culture medium was aspirated, and a volume of 500 µL *per* 1:2000 staining solution diluted in PBS was added to each well. The plates were kept for 10 minutes at room temperature and protected from light, after which the staining solution was washed three times with PBS.

Statistical analysis

Data are expressed as ± standard deviation (SD), comparing differences between groups was performed using the one-way ANOVA test followed by Dunett's multiple post-test comparisons. The used software was GraphPad Prism version 9.3.1 for Windows (GraphPad Soft-ware, San Diego, CA, USA, www.graphpad.com). The statistically significant differences between data were labelled with * (* $p < 0.1$; *** $p < 0.001$; **** $p < 0.0001$).

Results and Discussion

In plants, phenolic compounds are among the most abundant secondary metabolites. The biological activity of these plant compounds has been increasingly recognized in recent years [14].

Research in the field has highlighted their wide array of therapeutic effects, including antioxidant [16], anti-inflammatory [17], antimicrobial [18] and antitumour properties [19].

In order to identify the main polyphenols, present in the hydroalcoholic extract, LS-MS was used to quantify them. Accordingly, the table 1 below lists the main polyphenols found in extracts with a concentration greater than 3.5 µg/g d.m.

Table I

Individual phenolic compounds quantification by LC-MS in *Viscum album* extract

Standard compund	Rt (min)	Conc (µg/g d.m.)
Galic acid	4.868	4.706
Protocatechuic acid	11.132	3.622
Caffeic acid	21.035	16.172
Epicatechin	22.443	362.063
Ferulic acid	24.086	5.246
Rutin	25.765	46.712
Rosmarinic acid	28.650	33.383
Resveratrol	29.538	49.568
Quercetin	31.600	55.869
Kaempferol	34.761	181.481

Results indicate that epicatechin and kaempferol are both present in high concentrations. This result has also been confirmed by other researchers. Accordingly, Garcia-Garcia and collaborators highlighted the rich composition of mistletoe extract [20]. Moreover, Rahmawati *et al*., evaluated the composition of a mistletoe extract, identifying numerous active compounds such as epicatechin and quercetin, as well as rutin and other phenolic compounds [21].

Studies on the biological properties of *V. album* have primarily focused on the aqueous extract [22-25]. Nevertheless, there have been reports of ethanolic mistletoe extracts being used to treat cardiovascular disease and reduce blood pressure [26].

V. album has been the subject of numerous studies exploring its antitumour effects in various forms of extract. Aqueous extracts of *V. album* have been shown to have cytotoxic effects on a variety of cell lines, including lymphoma and squamous carcinoma of the tongue [27, 28]. Furthermore, the alcoholic extract from *V. album* showed antiproliferative effects in HeLa cells, without showing cytotoxic effects in healthy fibroblasts [29]. Similar studies have been conducted in which the ethanolic extract of *V. album* decreased the viability of colorectal cancer cells as well as melanoma cells in a dose-dependent manner. There is some evidence that the cytotoxic effect of mistletoe extracts is primarily due to altered mitochondrial activity and the cell cycle, but that other cellular processes may also be involved [9, 30]. Furthermore, *in vivo* studies have demonstrated the antitumour activity of alcoholic extracts and mistletoe glycerin, suggesting that stimulated immune mechanisms inhibit the proliferation of malignant cells [31]. Also, by combining *V. album* extract with doxorubicin, a synergistic effect was observed, which enhanced the antitumour effect at the level of Ehrlich tumour cells [32].

To determine the *in vitro* effect of mistletoe extract, two tumour cell lines were selected - murine melanoma and skin squamous cell carcinoma, as well as a healthy keratinocyte cell line, which was stimulated with five concentrations of VAex (50, 100, 250, 500 and 1000 µg/mL) for a period of 24 hours.

Regarding the effect of VAex on keratinocytes, there was a concentration-dependent decrease in cell viability. Thus, in the case of the lowest tested concentration (50 µg/mL), a slight decrease in cell viability was observed, at a value of approximately 96%. In contrast, at the highest concentration tested (1000 µg/mL), cell viability decreased by approximately 75% (Figure 1). In an earlier study, Kuonen *et al*. analysed the effect of a lipophilic extract of *Viscum album* on human fibroblasts and keratinocytes and found similar results. The lipophilic extract exhibits a more marked cytotoxic effect in human fibroblasts than in keratinocytes, according to the study. However, concentrations up to 200 µg/mL did not show cytotoxic effects on the HaCaT cell line [33].

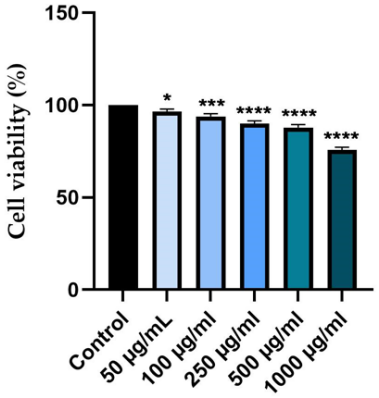

Figure 1.

In vitro assesment of the effect of VAex (50, 100, 250, 500 and 1000 µg/mL) exerts on the viability of keratinocytes (HaCaT) after 24 h of treatment

The data are expressed as viability percentages (%) normalized to control cells (untreated cells) and expressed as mean values ± SD of three independent experiments performed in triplicate. To identify the statistical differences between the Control and the nicotine-treated group, a one-way ANOVA analysis was conducted followed by the Dunett's multiple comparisons post-test (* $p < 0.1$; *** $p < 0.001$; **** $p < 0.0001$).

Regarding the impact of VAex on human keratinocyte nuclei, a concentration of 50 µg/mL

results in a slight condensation of chromatin, whereas a concentration of 1000 µg/mL results in the condensation and fragmentation of the nucleus as well. Both changes were indicative of cellular apoptosis (Figure 2).

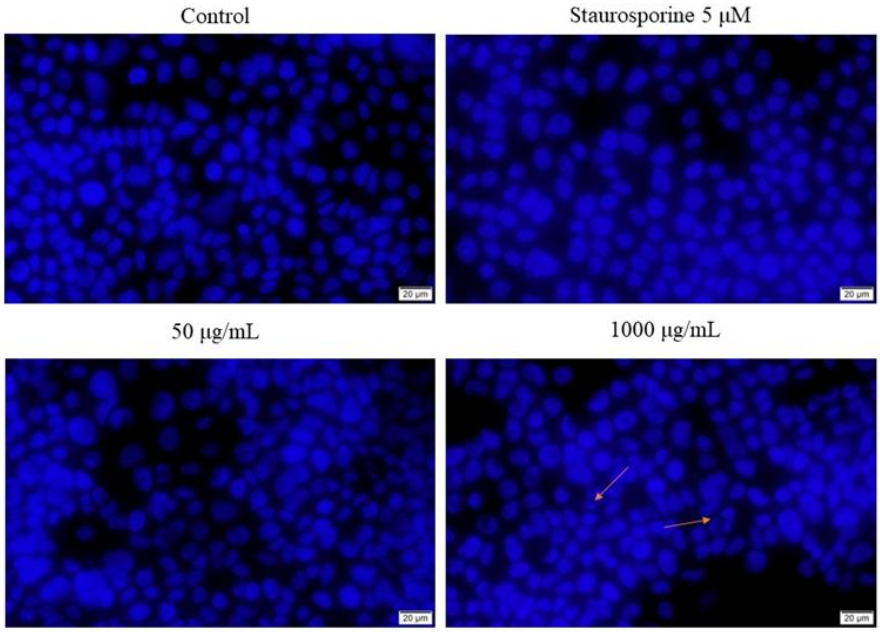

Figure 2.
Keratinocytes – HaCaT nuclei stained with Hoechst 33342 dye after a 24 h treatment with VAex (50 and 1000 µg/mL)
Staurosporine (5 µM) was used as the positive control for apoptotic changes at nuclear level. The orange arrows indicate signs of apoptosis. The scale bars represent 20 µm.

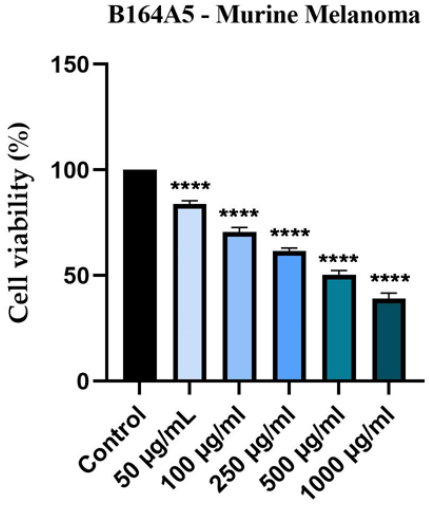

Figure 3.
In vitro assessment of the effect of VAex (50, 100, 250, 500 and 1000 µg/mL) exerts on the viability of murine melanoma cells (B164A5) after 24 h of treatment
The data are expressed as viability percentages (%) normalized to control cells (untreated cells) and expressed as mean values ± SD of three independent experiments performed in triplicate. To identify the statistical differences between the Control and the nicotine-treated group, a one-way ANOVA analysis was conducted followed by the Dunett's multiple comparisons post-test (**** $p < 0.0001$).

The same trend of decreasing cell viability in direct proportion to the tested concentration was observed in the case of tumour cells. Consequently, the murine melanoma cell line showed an obvious decrease in viability, the most noticeable effect being observed at the concentration of 1000 µg/mL, where the viability value was about 58%. However, even at low concentrations, cell viability was significantly lower than in keratinocytes (Figure 3). Han *et al*. evaluated the antimelanoma effect of a *V. album* extract, both *in vitro* and *in vivo*. In these studies, B16BL6 and B16F10 cell lines, as well as BDF1 mice, were used. The results showed that mistletoe extract causes G1/G0 arrest in both murine melanoma cell types. Furthermore, it has been shown that the extract induces cell apoptosis by increasing the activity of caspases involved in this process [34].

VAex has a significant effect on the nucleus of murine melanoma cells, even at a concentration of 50 μg/mL. Accordingly, at a concentration of 50 μg/mL, changes such as nuclear condensation and cell fragmentation were observed. In addition, at 1000 μg/mL, apoptotic bodies and massive chromatin condensation were noted (Figure 4). These results are in agreement with those which were obtained in the cell viability tests.

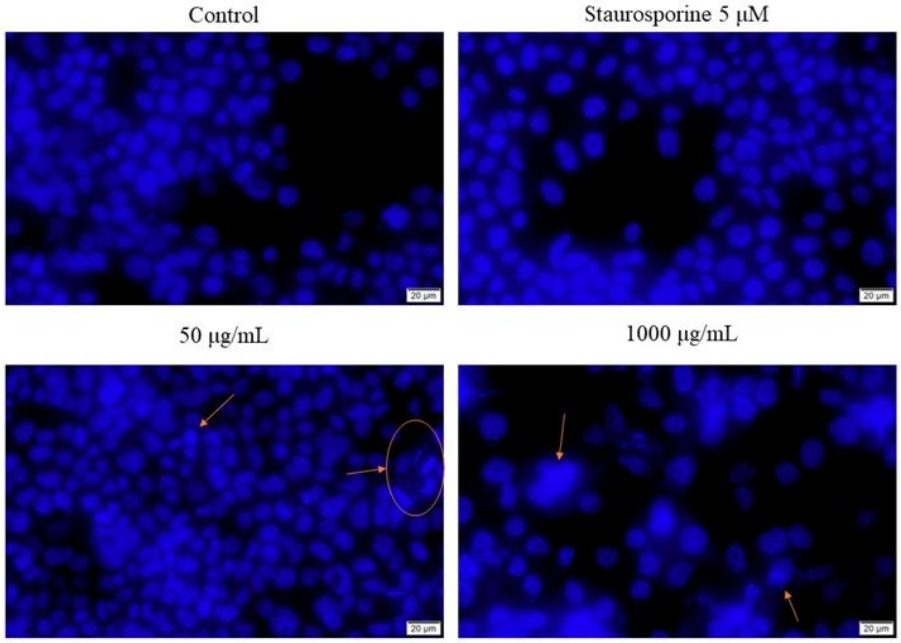

Figure 4.
Murine melanoma cells – B164A5 nuclei stained with Hoechst 33342 dye after a 24 h treatment with VAex (50 and 1000 µg/mL)
Staurosporine (5 µM) was used as the positive control for apoptotic changes at nuclear level. The orange arrows indicate signs of apoptosis. The scale bars represent 20 µm.

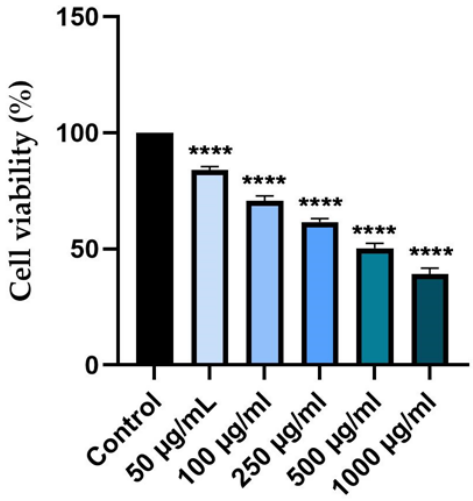

Figure 5.
In vitro assesment of the effect of VAex (50, 100, 250, 500 and 1000 µg/mL) exerts on the viability of skin epidermoid carcinoma cells (A431) after 24 h of treatment
The data are expressed as viability percentages (%) normalized to control cells (untreated cells) and expressed as mean values ± SD of three independent experiments performed in triplicate. To identify the statistical differences between the Control and the nicotine-treated group, a one-way ANOVA analysis was conducted followed by the Dunett's multiple comparisons post-test (**** $p < 0.0001$).

The most affected cell line was skin squamous cell carcinoma, where there were decreases in cell viability from 50 µg/mL (approximately 83%). And in this case, the most cytotoxic concentration was 1000 µg/mL, where the viability value was about 39% (Figure 5). A similar study evaluated the effect of *V. album* on the A431 cell line. The results of the study indicated that the mistletoe shows a similar study evaluated the effect of *V. album* on the A431 cell line. The results of the study indicated that mistletoe has antitumour properties by acting on angiogenic markers. antitumour properties by acting on angiogenic markers [35].

VAex manifests a strong effect on the nuclei of squamous skin carcinoma cells. Upon exposure to 50 µg/mL, apoptotic signals such as nucleus condensation are discernible, whereas at a concentration of 1000 µg/mL apoptotic bodies are formed and nuclei are condensed, as well as a decrease in number of nuclei compared to controls (Figure 6).

The results of the present study indicate that VAex has a strong cytotoxic effect in both pigmented (B164A5) and nonpigmented (A431) cells. The most affected cell line was squamous cell carcinoma, where VAex caused a decrease in cell viability in a dose-dependent manner and induced changes in the shape and structure of the nucleus, which indicate an apoptosis-like effect. Another important finding of the present study is that at low concentrations (< 500 µg/mL) VAex has a selective cytotoxic effect, the human keratinocyte cell line not being affected by stimulation for 24 hours.

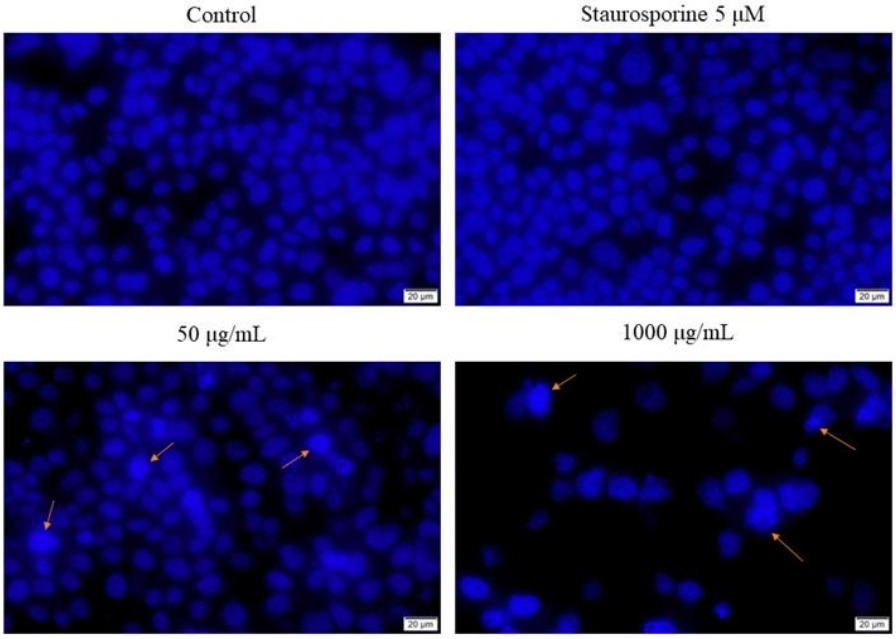

Figure 6.
Skin squamous cells carcinoma – A431 nuclei stained with Hoechst 33342 dye after a 24 h treatment with VAex (50 and 1000 µg/mL)
Staurosporine (5 µM) was used as the positive control for apoptotic changes at nuclear level. The orange arrows indicate signs of apoptosis. The scale bars represent 20 µm.

Apotosis is the natural process of cell death and is a potential therapy target for treatment of cancer. During the malignant process, apoptosis is inhibited by a number of biological changes, including the overexpression of antiapoptotic proteins and the under-expression of proapoptotic proteins. In turn, these changes result in intrinsic resistance to conventional anti-cancer therapy, such as chemotherapy. Current prospects for anticancer therapies are plant-derived compounds that are capable of activating the apoptotic pathway [36]. A study by Twardziok *et al.* demonstrated that mistletoe extract induced apoptosis in Erwing sarcoma cells. Mistletoe, according to the research group, has an antiproliferative effect and promotes apoptosis via intrinsic and extrinsic mechanisms, including activation of caspases 8 and 9 [37]. There has been similar evidence of *V. album* extract's pro-apoptotic effect in hepatocellular carcinoma and leukaemia [38-40].

Over time, polyphenols present in *V. album* extracts have been proven to be effective in antitumour therapy. Numerous compounds in this class have been studied for their ability to induce apoptosis in various types of cancer [41]. Epicatechin was one of the most abundant compounds found in *V. album* extract. Recent research has shown that it is a powerful antitumour agent. Thus, according to a study conducted by Pereyra-Vergara, epicatechin induces cell apoptosis in breast cancer cells [42]. The effect of various types of catechins found in green tea on melanoma has been studied. According to Zhang *et al*., catechins have anti-melanogenic effects in melanoma cells, with beneficial therapeutic effects [43].

In addition to the use of mistletoe extract as a therapeutic agent, its use in conjunction with conventional therapies can achieve a synergistic effect. Therefore, the association between *V. album* and mebendazole resulted in an increase in cytotoxicity of both compounds in the high-grade canine astrocytoma cell line [44]. Furthermore, the combination of *Viscum album* preparations with monoclonal antibodies lowers the risk of adverse effects associated with the use of monoclonal antibodies in antitumour therapy [45]. Additionally, Thronicke *et al.* observed a relationship between *Viscum album* and monoclonal antibodies, including trastuzumab, bevacizumab and rituximab. Based on the results of the study, it was concluded that the combination is therapeutically beneficial, as well as significantly reducing the rate of adverse effects associated with monoclonal antibody therapy [46].

Conclusions

According to preliminary results, VAex is cytotoxic toward pigmented and non-pigmented melanoma cells in a dose-dependent manner. As a result, 24 hours of stimulation led to a decrease in cell confluence, while, at the same time, morphological changes in the nucleus of the cells were observed, suggesting an apoptotic-like effect. A noteworthy finding of the study was that VAex is more

cytotoxic to non-pigmented cells (A431) than pigmented cells (B164A5). In addition, the polyphenol composition of mistletoe extract was also studied, and epicatechin appeared to be the most abundant component. Further research is needed to clarify the mechanism of action of mistletoe extract and to explain the influence of melanin on antimelanoma activity. Moreover, an important aspect of research on mistletoe hydroalcoholic extract is the possibility of combining it with conventional therapies, such as monoclonal antibodies, in order to enhance therapeutic effects and decrease side effects.

Conflict of interest

The authors declare no conflict of interest.

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