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Original Research

The extract of *Nicotiana glauca* induces apoptosis in rhabdomyosarcoma cells

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Abstract

Rhabdomyosarcoma, a malignant tumor commonly found in children and adolescents, often carries a poor prognosis. We conducted studies on the effects of the liposoluble extract of *Nicotiana glauca* (*N. glauca*), a plant from the Solanaceae family that is widely distributed, on the C2C12 murine myoblast cell line. We additionally investigated the antiproliferative effects of the extract on the RD rhabdomyosarcoma tumor cell line. The RD cells were pre-incubated without fetal bovine serum, followed by treatment with the liposoluble extract and the n-hexane sub-extract at specific concentrations. The effects on cell morphology were assessed using staining techniques. Cell viability, growth, and division capacities were evaluated through a wound assay. We analyzed the expression levels and subcellular localization of several proteins, including β-catenin, Notch 1, Caspase 3, and 14-3-3, using Western blotting. The extract of *N. glauca* induces apoptosis in C2C12 cells, suggesting that it may possess antitumor properties and could potentially be used in the treatment of hyperproliferative disorders. Our findings revealed that the control cells exhibited intact and normal nuclei, while the treated cells displayed characteristic morphological changes associated with apoptosis. Also, the treatments significantly reduced cell division capacity, and potentially cell migration, compared to the control group. Moreover, the evaluation of β-catenin protein localization showed a cytoplasmic and membrane proximity in C2C12 cells, whereas RD cells exhibited nuclear localization. These findings suggest the potential therapeutic value of the extract in targeting the proliferation and spread of rhabdomyosarcoma cells.

Keywords: Apoptosis, Rhabdomyosarcoma cells, Extracts of *N. glauca*

Introduction

Cancer is a leading cause of childhood mortality, with rhabdomyosarcoma (RMS) being the most prevalent type diagnosed in approximately two-thirds of pediatric cancer cases, particularly the embryonal subtype. RMS represents the most common soft tissue sarcoma in children and adolescents, accounting for around 2-3.5% of all pediatric malignancies [1]. The incidence of RMS is approximately four new cases per million children under the age of 20, and it is observed worldwide without a specific geographical predilection [2]. However, studies suggest a higher incidence among white individuals compared to black individuals [2]. RMS is characterized by its primitive mesenchymal origin and a propensity for differentiation into striated muscle tissue. While it can manifest in various anatomical sites, the extremities are the most common location in adults, followed by the trunk, genitourinary tract, head, and neck [3].

Histologically, RMS is classified into two subtypes: embryonal (eRMS) and alveolar (aRMS). eRMS is more prevalent in childhood and is commonly found in sites such as the orbit, head, neck, and genitourinary tract [2]. Recent studies have also reported a predilection for the upper and lower extremities [4]. On the other hand, aRMS constitute 20-25% of RMS diagnoses and is characterized by smaller, rounder cells resembling pulmonary alveoli. This subtype is more frequent in older children and displays a more aggressive behavior. Other less common variants of RMS include pleomorphic rhabdomyosarcoma (pRMS), which predominantly affects individuals older than 25 years and is associated with an unfavorable prognosis like aRMS; undifferentiated sarcomas, which comprise 11% of RMS cases and have a poor prognosis; and unclassifiable sarcomas, accounting for 5-10% of pediatric cases, which cannot be categorized due to the lack of defining characteristics and typically have a worse prognosis. In terms of immunochemical features, eRMS cells exhibit nuclear localization of β-catenin in addition to the typical cytosolic and proximal membrane localization. They also express high levels of N-cadherin and integrin α-9, both of which are positively regulated by Notch pathway. These molecular characteristics enhance cell mobility, invasiveness, aggressiveness, and the maintenance of an undifferentiated state within the tumor [5]. Cancer cells gradually acquire distinct biological capabilities, referred to as "hallmarks," enabling tumor establishment and progression [6,7]. These hallmarks include the activation and maintenance of proliferative signals, evasion of growth suppressive signals, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion mechanisms and metastasis (such as aberrant Wnt-β-catenin signaling with low E-cadherin levels and nuclear localization of β-catenin), reprogramming of energy metabolism to support continuous cell proliferation, and evasion of immune system surveillance. Genomic instability and mutations in neoplastic cells are key facilitators of cancer hallmark acquisition, generating genetic variations responsible for these distinct capabilities. Inflammation, which contributes bioactive molecules to the tumor microenvironment, is another facilitating characteristic of tumor development [7].

Apoptosis, an active process in normal skeletal muscle during intense exercise or in pathologies such as muscular dystrophy, muscle denervation, myopathies, and sarcopenia, is of interest in RMS research. These conditions are often associated with the accumulation of defective mitochondria [8] or reduced oxidative capacity due to mitochondrial dysfunction [9]. Our previous studies demonstrated that C2C12 myoblasts share similarities with activated satellite cells surrounding mature myofibers, as they proliferate and differentiate, participating in tissue repair during cellular injury. We also identified hydrogen peroxide (H_2O_2) as an inducer of apoptosis in C2C12 cell line, observing characteristic morphological changes. Additionally, we investigated the apoptotic pathway components activated during apoptosis, including proteins, transcription factors, and regulated genes. We observed a reduction of these effects upon treatment with 17β-estradiol (E2) or testosterone, suggesting their potential protective roles [10-16]. Furthermore, we found that the liposoluble extract of *Nicotiana glauca* (*N. glauca*) induces apoptosis in C2C12 cells, involving caspase activation and the regulation of pro- and anti-apoptotic protein gene expression. In contrast, the aqueous extract of *N. glauca* had no effect on the cells [17]. In this study, we aim to further investigate the effects of *N. glauca* extracts on RD cells, including viability, replication, migration, and the expression and subcellular localization of key proteins involved in invasion, metastasis, and apoptosis pathways.

Materials and Methods

Materials

Mito Tracker *(MitoTracker* ® Red CMXRos) was from Sigma Aldrich, Alexa Fluor 488-conjugated anti-mouse secondary antibody was from Molecular Probes (Eugene, OR, USA). Anti-Lamin B (B-10): sc-374015, anti β-catenin (H-1): SC-133240, anti-caspase 3, and anti-Notch 1 (mN1A): sc-32745 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other reagents used were of analytical grade.

Cell culture

C2C12 (ATCC number: CRL-1772™) mouse myoblast cell line and human rhabdomyosarcoma RD cell line (ATCC number: CCL-136TM) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 5% CO_2 and passaged were performed every 48 to 72 hours using fresh medium.

Nicotiana glauca extracts

N. glauca plants specimens were collected from their natural habitats in Buenos Aires Province, Argentina and were grown under greenhouse conditions. An herbarium voucher specimen has been kept at the Herbarium of the Department of Agronomy Universidad Nacional del Sur from *N. glauca* leaves, the liposoluble (crude) and *n*-hexane sub - extracts were obtained. The liposoluble extract was isolated following the Bligh & Dyer method [20]. The starting plant material was initially homogenized in chloroform - methanol (1:2, v/v) using a refrigerated Sorvall centrifuge at 8°C at maximum speed for 2 minutes. The samples were further homogenized after the addition of chloroform (1.2 ml/g of tissue) for 30 seconds and then after the addition of distilled water (1.2 ml/g) for another 30 seconds. The final homogenate was centrifuged at 4300 x g for 20 minutes. The lower liposoluble phase was collected and evaporated by flushing nitrogen at 35°C. The final residue was solubilized in isopropanol. The concentrated liposoluble extract (not lyophilized) was partitioned into three sub-extracts: *n*-hexane, chloroform, and ethyl acetate. Among these three sub extracts, the *n*-hexane sub extract had the highest apoptotic effect in muscle cells, determined in cell cultures using the TUNEL assay and based on the changes induced in cell morphology, visualized by the staining with DAPI and *MitoTracker red* (MTT) [17]. The extracted solutions were evaporated under reduced pressure and then lyophilized.

Extract preparation

The extract, 0.1 mg dry powder, was resuspended in 500 µl of isopropanol (stock). Dilution preparation: 1 µl of the stock was resuspended in 1000 µl of serum-free DMEM (1:1000) and from this first dilution the final dilution was prepared: 1:1000 in serum-free DMEM medium [17,18]. At this concentration, it was proved that the *n*-hexane sub extract induces a 70% of apoptosis [19].

Cells treatments

The treatments were performed with 70-80% confluents cultures in medium without serum for 20 minutes. Then, treatments were carried out by adding the liposoluble and *n*-hexane extract of *N. glauca* or the vehicle, isopropanol (IPA) (the IPA percentage in the culture medium assay, of the cells treated with extracts or the vehicle alone, was less than 0.001%) during 1 or 2 hours at 37°C in a humid atmosphere of 5% CO_2 in air. Cells were cultured in 3 cm and 6 cm sterile plates (Greiner Bio-One, Frankenhauser, Germany).

Mitotracker red (MTT) and DAPI staining

After treatment, the cells attached to the coverslips were stained with MTT, which was prepared on dimethyl sulfoxide (DMSO) and added to the cell culture medium at a final concentration of 1 µmol/L (1:10000, prepared in DMEM without FBS). After 30 minutes incubation at 37°C, the cells were washed with PBS 1X (pH 7.4, 8 g/L NaCl, 0.2 g/l KCl, 0.24 g/L KH_2PO_4, 1.44 g/L Na_2HPO_4) and they were fixed with methanol at -20°C for 30 minutes. Cells were washed 3 more times with PBS 1X to ensure that any methanol residue had been removed. The cells were later stained with DAPI (1:500 dilution from a stock solution of 5 mg/ml). This was incubated for 30 minutes at room temperature in darkness and gentle stirring. The cells were then washed 3 times with PBS after the incubation time, and the samples were prepared for viewing under the microscope. The cells were examined using a fluorescence microscope (NIKON Eclipse TiS) equipped with standard filter sets to capture fluorescent signals. Images were collected using a digital camera.

Wound scratch assay

Cells were seeded in dishes and cultured until reaching 80-90% confluence. The treatments with *N. glauca* extracts were performed, and after the specified time, a cross-shaped wound was made on the cell monolayer. The medium was changed to fresh medium, and the wound closure was monitored for 24 hours or until complete closure. Images were taken at regular intervals using a digital camera coupled to an optical microscope (Nikon Eclipse Ti-S).

Immunocytochemistry

After treatments, semi-confluent (70-80%) monolayers were washed with serum-free phenol red-free DMEM and then fixed and permeabilized for 20 minutes at -20°C with methanol to allow intracellular antigen labeling. After fixation, cells were rinsed 3 times with PBS. Non-specific sites were blocked for 30 minutes in PBS that contained 5% bovine serum albumin. Cells were then incubated overnight at 4°C, in the presence or absence (negative control) of primary antibodies (1:50 dilution). The primary antibodies were recognized by fluorophore-conjugated secondary antibodies. Finally, the coverslips were analyzed by conventional fluorescence microscopy.

Subcellular fractionation

RD monolayers were scrapped and homogenized in ice cold Tris - EDTA - sucrose (TES) buffer (50 mM Tris-HCL (pH 7,4), 1 nM EDTA, 250 mM sucrose, 1 mM DTT, 0,5 mM PMSF, 20 mg/ml leupeptin, 20 mg/ml aprotinin, 20 mg/ml trypsin inhibitor) using a Teflon - glass hand homogenizer. Total homogenate was centrifuged at 100 x *g* for 5 min at 4°C to eliminate the unbroken cells, partially disrupted cell, and other debris. Then, total homogenate free of debris was used to obtain the different fractions (nuclear, mitochondrial, and mitochondrial supernatant). The nuclear pellet was obtained by centrifugation at 300 x *g* during 20 min at 4°C. The supernatant was further centrifuged at 10000 x *g* for 30 min at 4°C to yield the mitochondria pellet. The remaining solution was called mitochondrial supernatant. Pellets were resuspended in lysis buffer (50 mM Tris-HCL, pH 7,4, 150 mM NaCl, 0,2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM PMSF, 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Protein concentration of the fractions was estimated by the method of Bradford [21] and Western blot assays were performed. Cross contamination between fractions was assessed by immunoblots using antibodies against anti - Lamin B as nuclear markers.

Western blot analysis

Protein aliquots (25 µg) were combined with sample buffer (400 mM Tris-HCL (pH 6.8), 10% SDS, 50% glycerol, 500 mM dithiothreitol (DTT), and 2 mg/ml Bromophenol Blue, boiled for 5 min and resolved by 10% SDS-PAGE according to the method of Laemmli [22]. Fractionated proteins were then electrophoretically transferred onto Polyvinylidene Difluoride (PVDF) membranes (Immobilon-P; Millipore, Darmstadt, Germany), using a semi-dry system. The nonspecific sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T). Blots were incubated overnight with the appropriate dilution of the specific primary antibodies against the proteins studied. The membranes were repeatedly washed with PBS-T prior to incubation with horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescence (ECL) blot detection kit (Amersham, Buckinghamshire, England) was used as described by the manufacturer to visualize reactive products. Relative quantification of Western blot signals was performed using ImageJ Software (NIH, USA).

Statistical analysis

Results are shown as means ± S.E.M. Statistical differences among groups were determined by ANOVA followed by a multiple comparison post hoc test, the Di Rienzo, Guzma´n Casanove´s (DGC) test [23]. Data are expressed as significant at P<0.05.

Results

The liposoluble extract of *N. glauca* induced morphological changes typical of apoptosis in RD cells

As a first approach to investigate the effects of the *N. glauca* extract on the apoptosis of RD cells, mitochondrial and nuclear morphology were studied by immunocytochemistry assays and immunofluorescence conventional microscopy using Mitotracker and DAPI dyes, which were performed after treatment. It was observed that the liposoluble extract of *N. glauca* induced apoptosis on RD cell line, as evidenced by changes in nuclear and mitochondrial morphology. Under control conditions, muscle cells exhibited intact/normal nuclei and a "spider web" distribution of mitochondria. However, treatment with the liposoluble extract replicated the characteristic morphological alterations observed in apoptotic cells, including nuclear condensation and loss of the typical mitochondrial distribution **(Figure 1)**.

Extracts from *N. glauca* affected the cell cycle by reducing the rate of cell division and potentially also influencing cell migration

The effects of the extracts of *N. glauca* on the cell cycle and cell migration were then analyzed. After reaching 100% confluence, RD cells were treated for 1 hour with either *n*-hexane or the liposoluble extract. A cross-shaped wound was then created, and the closure of the central area of the wound was monitored every 20 minutes under a light microscope. **Figure 2** shows the images captured at zero hours (after creating the wound and replacing the medium with serum-containing culture medium, 0% of wound occupancy) and at 24 hours (end of the assay). The treatment with the liposoluble extract and the *n*-hexane sub-extract significantly inhibited the division capacity and likely reduced cell migration (3% and 1% of wound occupancy, respectively) compared to the control, where complete

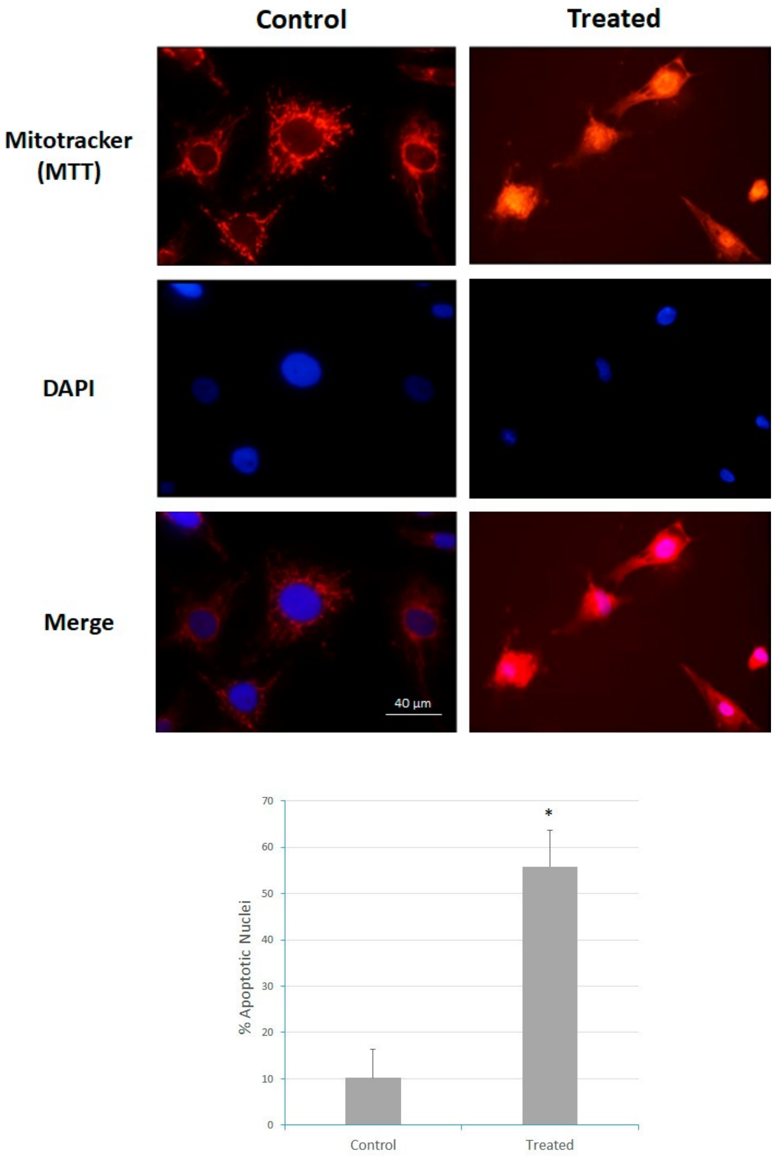

Figure 1. The liposoluble extract of *N. glauca* induces apoptosis in RD cells. Cell cultures were treated with the liposoluble extract of *N. glauca* for 2 h (Treated) or maintained in DMEM without serum for 2 h (Control). Cells were then stained with MTT Red (red) and subsequently fixed and stained with DAPI (blue), as described in the Methods section. Morphological analysis of fluorescence-stained nuclei and percentages of apoptotic cells at each condition are shown. At least ten fields per dish were examined. Each value represents the mean of three independent experiments. Averages ± S.E.M. are given; *P <0.05 with respect to the control. Magnification: 63X.

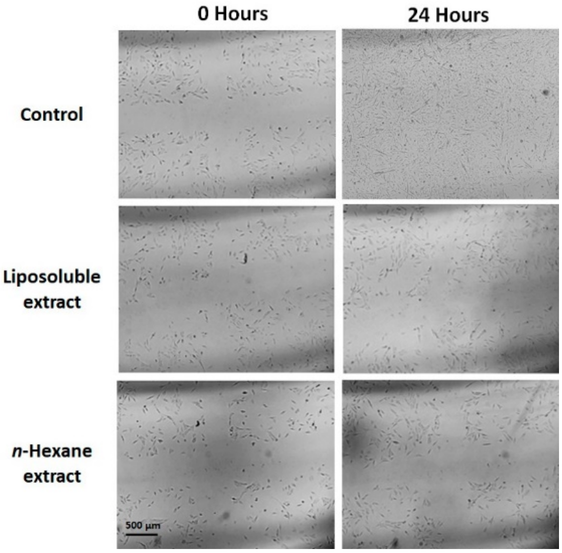

Figure 2. Extracts from *N. glauca* affect the cell cycle reducing the rate of cell division and possibly also cell migration. Cells were incubated in sterile 6 cm dishes until 100% confluence. After the treatments, a cross-shaped wound was made, and the medium was replaced by fresh culture medium containing serum (0 hours). Wound closure was monitored for 24 hours. 0 hours: start of monitoring. 24 hours: completion of the trial. Magnification: 4X.

wound closure was observed 24 hours after the start of the assay (95% of wound occupancy) **(Figure 2).** These findings indicate that *N. glauca* extracts also impact the cell cycle, resulting in a reduced rate of cell division and potentially affecting cell migration.

N. glauca extracts induced the nuclear localization of β-catenin protein in RD cell line

As a first approach to investigate the effect of *N. glauca* extracts on the subcellular localization of β-catenin, we initially studied its localization under basal conditions in the RD cell line, comparing results with the non-tumor C2C12 cell line. Thus, immunocytochemical assays were performed using a specific antibody against β-catenin to evaluate its localization in both RD and C2C12 cells. We observed that non-tumor cells exhibited a cytoplasmic localization of β-catenin near the cell membrane, whereas RD cells showed nuclear localization **(Figure 3)**. Immunocytochemistry after treatments, revealed a change in the subcellular localization of β-catenin in the RD cell line after exposure to the liposoluble extract and *n*-hexane sub-extract. Untreated RD cells showed nuclear β-catenin localization, whereas treated cells exhibited increased cytosolic localization **(Figure 4A)**. Western blot analysis further confirmed these effects, showing that, although no changes were observed in the nuclear fraction, an increase in β-catenin localization was detected in the post-mitochondrial fraction following treatment with both the liposoluble extract and the n-hexane sub-extract **(Figure 4B)**. Lamin B detection was used as a control to verify the absence of nuclear contamination in the post-mitochondrial fraction.

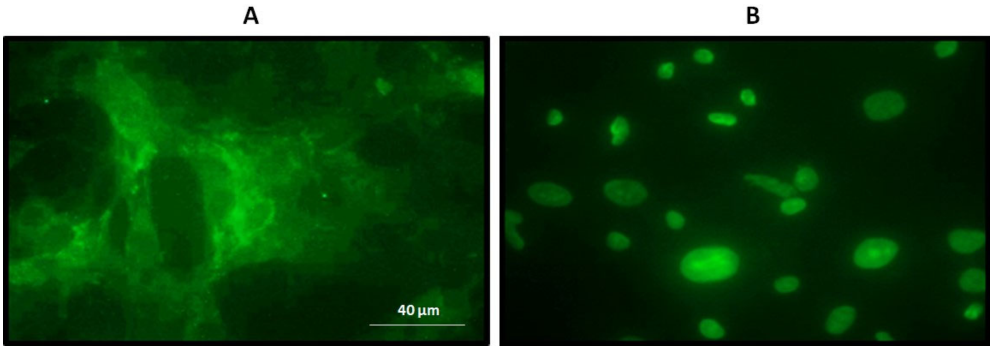

Figure 3. β-catenin is localized in the nucleus of RD tumor cells. Immunocytochemistry assays using a primary antibody specific against β-catenin and then a secondary conjugated to a fluorophore (Alexa 488). **A.** C2C12 cells: β-catenin with cytoplasmic and membrane-proximal localization. **B.** RD cells: β-catenin with nuclear localization. Magnification: 63X.

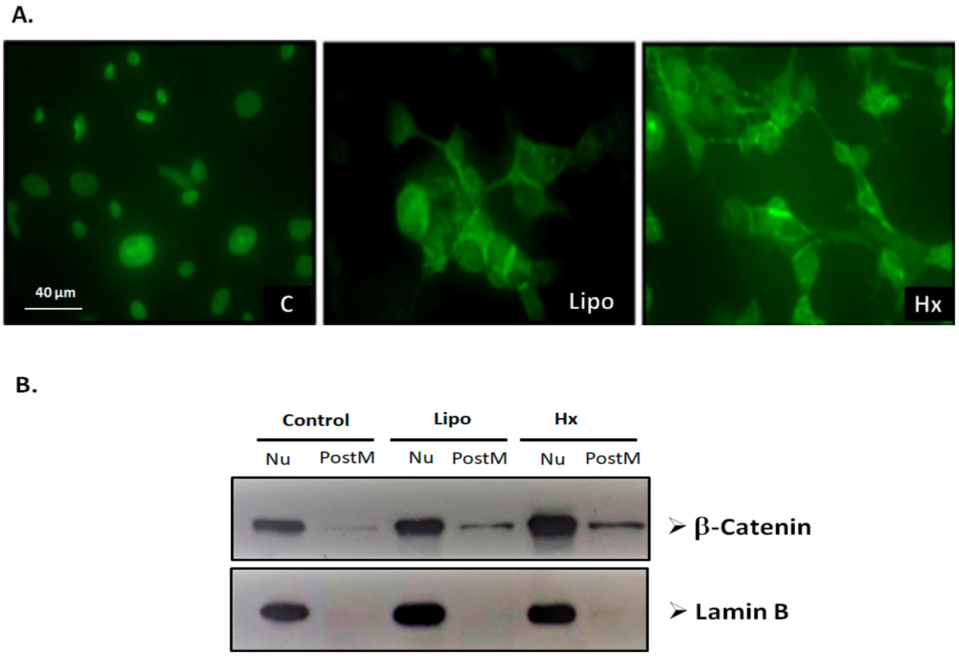

Figure 4. Extracts from *N. glauca* induce changes in the subcellular localization of β-catenin protein in RD cells. A. Cells cultured on coverslips were treated for 1 hour with the vehicle of the extracts (C), with the liposoluble extract (Lipo) or with the *n*-hexane sub extract (Hx). Cells were fixed, permeabilized, and incubated with a primary antibody specific for β-catenin and a fluorophore-labeled secondary antibody. Nuclear localization was observed in the control (C). In the cells treated with the extracts (Lipo and Hx), β-catenin was detected in the nucleus and cytosol, mainly located near the plasmatic membrane. Magnification: 63X. **B**. The subcellular fractions obtained after the different treatments were isolated and quantified. 20 µg of protein from each fraction were analyzed by Western blot assays. The membranes were incubated using a specific antibody against β-catenin and subsequently with the peroxidase-conjugated secondary antibody, as detailed in Methods. The nuclear fraction (Nu) and the post-mitochondrial fraction (PostM) were evaluated under control conditions (C), liposoluble extract (Lipo) and *n* hexane sub extract (Hx).

N. glauca extracts reduced cleavage and nuclear localization of the intracellular fragment of Notch 1 in RD cells

Immunocytochemical assays using a specific antibody against the intracellular fragment of Notch 1 were performed to assess its localization under both basal conditions and after treatment. Under basal conditions, the intracellular fragment of Notch 1 was predominantly localized in the nucleus **(Figure 5A).** Treatment with the liposoluble extract decreased the nuclear localization of the fragment (**Figure 5B)**, while treatment with the *n*-hexane sub-extract resulted in its localization near the plasma membrane **(Figure 5C)**. These findings indicate that *N. glauca* extracts reduce the cleavage and nuclear translocation of the intracellular region of the Notch 1 protein in the RD cell line.

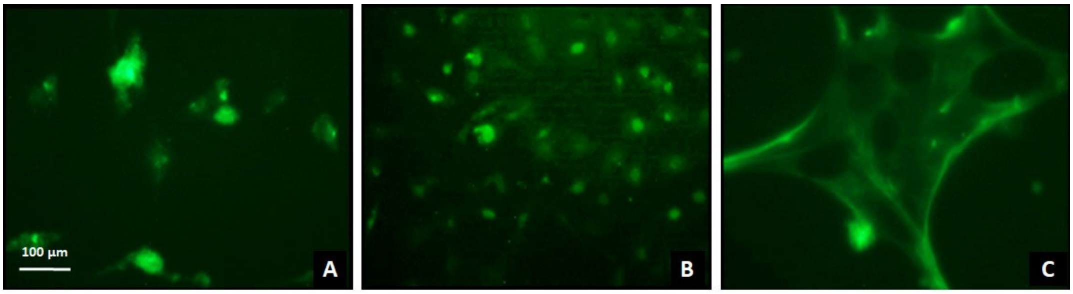

Figure 5. Treatment with *N. glauca* extracts reduces cleavage and nuclear localization of the intracellular fragment of Notch 1 in RD cells. Cells were seeded on coverslips, treated with the liposoluble extract (B), the *n*-hexane sub-extract (C) or untreated (A) and subsequently fixed and permeabilized with methanol. A primary antibody directed specifically against the intracellular region of Notch 1 and a secondary antibody conjugated to the Alexa 488 fluorophore were used, as described in Methods. Magnification: 20X.

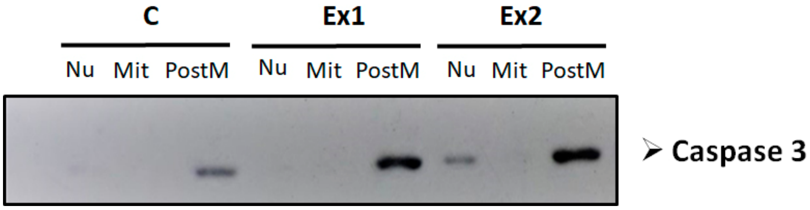

Figure 6. The liposoluble extract of *N. glauca* increases the cleaved (active) form of Caspase 3 and its nuclear localization. The subcellular fractions obtained after the different treatments were isolated and quantified. 20 µg of protein from each fraction were analyzed by Western blot assays. The membranes were incubated using a specific antibody against the active form of Caspase 3. The nuclear (Nu), mitochondrial (Mit) and the post-mitochondrial fraction (PostM) obtained from cells treated with the liposoluble extract for 1 hour (Ex 1), 2 hours (Ex 2), and cells maintained in DMEM (C) were analyzed.

The liposoluble extract of *N. glauca* increases the cleaved form of Caspase 3 and its nuclear localization

Western blot assays using a specific antibody against the cleaved (active) form of the proapoptotic protein Caspase 3 demonstrated an increased detection of the cleaved fragment in the post-mitochondrial fraction, followed by its translocation to the nucleus in a time-dependent manner (**Figure 6**), being its nuclear localization observed after 2 hours of treatment with the liposoluble extract. These findings indicate that *N. glauca* extracts increase the activation and nuclear localization of Caspase 3 in RD cell line.

Discussion

One of the most prominent characteristics of cellular changes during apoptosis is nuclear condensation, which can be effectively observed by staining DNA with dyes such as DAPI. DAPI is a fluorescent marker that binds strongly to DNA, particularly in adenine-thymine-rich regions. It has an absorption maximum at a wavelength of 358 nm (ultraviolet) and an emission maximum at 461nm (blue). In fluorescence microscopy, DAPI is excited with ultraviolet light and detected through a blue/cyan filter. In non-apoptotic cells, DAPI-stained nuclei typically appear rounded and well-defined. However, during apoptosis, the nuclei undergo pyknosis, characterized by chromatin condensation and DNA fragmentation. As a result, the nuclei become compacted and often lose nuclear material, deviating from their typical rounded shape. These apoptotic nuclear morphological changes were clearly observed in RD cells treated with the liposoluble extract of *N. glauca*, further supporting the extract's role in inducing apoptosis. Fluorescence microscopy was also employed to assess mitochondrial morphology and distribution, which serves as an additional indicator of apoptosis. Mitochondria were specifically labeled using the fluorescent dye MTT red, as described in the *Methods* section. We could clearly observe that treatment of RD cells with the liposoluble extract caused a significant disruption in mitochondrial distribution. In untreated cells, mitochondria typically exhibit a homogeneous "spider web" pattern distributed throughout the cytoplasm. However, after treatment with the liposoluble extract, this arrangement was altered, with mitochondria clustering around the nucleus, a well-documented hallmark of apoptosis. Therefore, the observed changes in mitochondrial morphology and distribution further support the notion that treatment with the liposoluble extract of *N. glauca* induces apoptosis in RD cells. To evaluate the effects of the lipid extract and the n-hexane sub-extract on cell proliferation and migration in RD cells, a wound assay was performed. Cells were cultured in complete medium until they reached 100% confluence. Afterward, a cross-shaped wound was created as detailed in the *Methods* section. The cells were treated with the extracts, and the wound closure progress was monitored over 24 hours. Remarkably, we observed that cells treated with the lipid extract and the n-hexane sub-extract exhibited a significantly reduced ability to cover the wounded area compared to the control group, which demonstrated efficient wound closure. This observation indicates that both extracts impeded the proliferation and possibly the migration of RD cells, supporting the potential of these treatments to inhibit tumor cell spread and growth. We proceeded to investigate the expression levels and subcellular localization of several proteins that play crucial roles in the migratory and invasive capabilities of RMS. Additionally, we examined the expression, activation, and subcellular localization of Caspase 3, a key protein involved in the final phase of the apoptotic cascade. For this analysis, we used specific primary antibodies targeting β-catenin, the cleaved fragment of Notch 1 receptor and the cleaved fragment of Caspase 3. These proteins were selected for their important roles in the progression of RMS (β-catenin and Notch 1) and apoptosis (Caspase 3). Upon evaluating the β-catenin and Notch 1 proteins, significant changes in their subcellular location were observed following the treatments with *N. glauca* extracts compared to the control group. Notch 1 intracellular fragment exhibited a pronounced loss of their nuclear localization, particularly after *n*-hexane sub-extract treatment, while maintaining their membrane labeling (indicative of the inactive form of the receptor). In the case of β-catenin, immunocytochemistry showed a notable increase in cytosolic localization, especially near the cellular membrane, following the treatments. This contrasts with the nuclear localization typically observed in untreated RD cells. Both the intracellular fragment of Notch 1 and β-catenin interact with proteins that function as transcription factors. The association of Notch 1 intracellular fragment and β-catenin with their respective transcription factors leads to conformational changes and subsequent activation of the specific transcription factors, thereby regulating the expression of genes under their control.

The cleaved Notch1 fragment plays a pivotal role in regulating genes like N-cadherin and integrin α-9, both of which are crucial for cell migration and invasion events. The presence of integrin on the plasma membrane facilitates juxtacrine interactions with fibronectin in the extracellular matrix, enabling migration until reaching a blood capillary. At that point, N-cadherin interacts with N-cadherin on the vascular endothelium, allowing RMS cells to enter and exit the bloodstream during invasion and metastasis. Integrin α-9 plays a vital role in maintaining the malignant phenotype and the immature state of the muscle lineage [5]. RMS, as mentioned before, is characterized by the activation of a common mechanism that promotes cell adhesion, migration, and invasion, thereby contributing to its oncogenic properties. This mechanism is mediated by the Notch pathway, which positively regulates the expression of key molecules such as N-cadherin and integrin α-9. N-cadherin is a calcium-dependent adhesion molecule primarily involved in cell adhesion and migration processes. It is prominently observed during embryonic development, in undifferentiated cell stages, and throughout differentiation. The increased expression of N-cadherin and integrin α-9 is believed to play a significant role in the oncogenicity of the Notch pathway in RMS [5]. This upregulation of protein expression contributes to the aberrant adhesion, migration, and invasion capabilities exhibited by RMS cells, key features associated with the aggressive nature of this malignancy. The activation of apoptosis leads to significant changes in cell morphology, including cytoskeletal disorganization, altered mitochondrial distribution, and nuclear fragmentation. These changes disrupt the typical cellular localization of various proteins crucial for the development of tumorigenic capacities. Notably, the nuclear localization of the cleaved fragment of Notch1 plays a vital role in regulating the expression of N-cadherin and integrin α-9. These proteins are essential for the migration, intravasation, and metastasis of RMS cells. The nuclear localization of β-catenin, which is facilitated by the activation of the Notch pathway, also positively regulates the expression of genes associated with migration and invasion. Consequently, the decrease or loss of nuclear localization of these proteins significantly impacts the properties of the RD cell line, disrupting signaling pathways involved in tumor progression and compromising the cells´ migratory and invasive abilities. During apoptosis, changes in cellular machinery and subcellular structures have a direct impact on the behavior of proteins that would normally be active or inactive in a non-apoptotic state. These changes encompass alterations in activation states, which affect conformational changes such as phosphorylation or dephosphorylation and interactions with other molecules or proteins. Additionally, changes in subcellular localization contribute to the modification of cellular properties observed prior to the apoptotic state. In the context of the proteins studied in this work, the loss or reduction of nuclear localization for both Notch1 and β-catenin represents a significant alteration in cells' oncogenic properties. This is due to the decreased expression of genes involved in critical tumor progression events regulated by these factors. The observed changes in protein localization and activity during apoptosis disrupt normal signaling pathways associated with cell migration, invasion, and metastasis. Consequently, the cellular properties and capabilities that contribute to the malignant behavior of the RD cell line are profoundly affected. Caspase 3 plays a crucial role in mediating the morphological and biochemical changes observed in apoptotic cells. As one of the main caspases involved in apoptosis, Caspase 3 targets various substrates to orchestrate the dismantling of cellular components. One of its key substrates is a factor responsible for DNA fragmentation, leading to the characteristic DNA degradation observed in apoptotic cells. Additionally, caspases contribute to the elimination of polymerase, an enzyme involved in DNA damage repair processes. Beyond its role in DNA fragmentation and repair inhibition, Caspase 3 activation triggers pathways leading to chromatin condensation, contributing to nuclear compaction. Moreover, Caspase 3 participates in the degradation of nuclear lamina and cytoskeleton proteins, further aiding in the restructuring of the cell during apoptosis [24]. By performing Western blot assays, an increase in the cleaved fragment of Caspase 3 was observed in RD cells treated with the liposoluble extract of *N. glauca*. This finding suggests that the extract induces apoptosis in RD cells, as evidenced by the activation of Caspase 3 and the generation of its cleaved fragment. The demonstrated apoptotic effect of the extracts is not limited to RD cells, as mentioned in the Introduction. These extracts have also been shown to affect the proliferative capacities of other cell lines, such as the non-tumor cell line C2C12, ultimately inducing apoptosis in these cells as well. This broader impact on different cell lines underscores the potential therapeutic relevance of the extracts. Given the promising apoptotic activity exhibited by the extracts, our research group aims to further investigate and elucidate the molecular components responsible for these effects. Our future objective involves the purification and characterization of the active molecules within the extracts. By conducting comprehensive analyses, we aim to identify the specific molecules that not only reduce or eliminate cell proliferation, migration, and invasion but also have the potential to induce cell differentiation. The discovery of such molecules holds significant importance as they may offer novel therapeutic strategies for the treatment of various conditions, including cancer. The ability to induce cell differentiation is particularly valuable, as it can provide an alternative approach to combat the uncontrolled growth and malignant behavior of tumor cells. Through continued research and analysis, we endeavor to uncover the precise molecular mechanisms underlying the apoptotic and differentiating properties of the extracts, with the goal of contributing to the development of effective therapeutic interventions.

Conclusions

In conclusion, the study focused on the cellular changes associated with apoptosis, particularly in RD cells treated with extract of N. *glauca*. First, nuclear condensation, a prominent characteristic of apoptosis, was observed through DNA staining with DAPI. This staining revealed significant morphological changes in cell nuclei, deviating from their usual rounded and well-defined shape, indicative of apoptosis. Second, mitochondrial morphology and distribution were assessed using MTT red fluorescence dye. Treatment with the extract disrupted the typical mitochondrial organization, causing mitochondria to gather around the nucleus, a hallmark of apoptosis. Additionally, a wound assay demonstrated that the extract inhibited the proliferation and migration of RD cells, suggesting potential therapeutic implications. The study also explored the expression and subcellular localization of key proteins, notably β-catenin, Notch1, and Caspase 3. The treatments induced significant changes in the subcellular location of these proteins, impacting their functions in signaling pathways related to cell migration, invasion, and apoptosis. Notch1 and β-catenin play critical roles in regulating genes associated with cell migration and invasion, and their altered localization during apoptosis disrupted these pathways, affecting the oncogenic properties of RD cells. The Caspase 3 activation was also observed, further confirming the induction of apoptosis by the extract. The study's future goal is to identify and characterize the specific molecules within the extracts responsible for these effects, with the aim of developing novel therapeutic strategies, particularly those that trigger cell differentiation to counteract uncontrolled growth and malignant of these cells.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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