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Extracts from *Nicotiana glauca* induce apoptosis through caspases in skeletal muscle cells

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Abstract: Phytoestrogens are plant compounds which have generated considerable interests. A litany of health benefits including a lowered risk of osteoporosis, breast cancer, and menopausal symptoms, are frequently attributed to phytoestrogens but without the knowledge of their side effects. We investigate the effects of lipid extracts from the Solanaceae *Nicotiana glauca* on skeletal muscle cells, in relation to apoptosis. Opposite to the effects of 17β-estradiol, the crude extract from *N. glauca* and its sub-extracts induced apoptosis in C2C12 cells. This apoptotic action involved caspase 3/7 activation. These data suggest that the traditional use of this medicinal plant could affect the skeletal muscle homeostasis.

Keywords: phytoestrogens, apoptosis, skeletal muscle, caspase 3/7, *Nicotiana glauca*, Solanaceae.

1. Introduction

In recent years, plants have gained many interests for being the vital sources of the discovery of pharmacologically active compounds [1,2]. Compounds from higher plants also called phytochemicals, serve as a source of new drugs for treating diverse forms of diseases. However, several pharmacological compounds from plants have been traditionally used without the actual knowledge of their side effects. In addition, the molecular mechanisms by which plant-derived compounds exert their effects are not yet fully understood.

Among diverse groups of phytochemicals, phytoestrogens have generated considerable interests as alternatives for hormone replacement therapy (HRT) or due to its preventative or therapeutic actions in carcinogenesis, atherosclerosis, and osteoporosis [3].

The phytoestrogens were first identified in the 1940s [4]. These plant compounds have got structural similarities with mammalian 17β-estradiol (E2) and are capable of binding to estrogen receptors (ERs) [5-7]. Even though phytoestrogens can activate ERs, their effects are approximately thousand folds weaker than E2 [8]. They have been shown to possess estrogen like activity as they alleviate postmenopausal complaints, increase bone formation and repress adipose tissue similar to E2 [9]. Although there are many positive indications that phytoestrogens can fulfil those actions, it remains to be proven: controlled interventional studies are lacking, many side effects have not been evaluated and many questions remain unanswered.

As mentioned, the synthesis of molecules structurally and functionally related to the mammalian steroid hormones by plants, has been reported [5,6,10,11]. However, their physiological role in plants is controversial. Various steroids derived from plant and animal sources have been

shown to affect cell growth and tissue differentiation in vascular plant systems. The existence of receptor-like molecules as those of animal cells could provide clues into a possible steroid mechanism of action.

In our previous works, we determined the relative contents of E2, estrone and progesterone like-molecules in *Solanum glaucophyllum* Desf. *(Solanum malacoxylon* Send.*)*, *Lycopersicon esculentum* (Mill) and *Nicotiana glauca* (Graham) by competition assays using specific polyclonal antibodies against the respective mammalian steroid hormones [5,6,12]. Binding experiments with [^{3}H]17β-estradiol in the presence or absence of an excess of the unlabelled steroid showed that the Solanaceae organs contain estrogen binding sites. The protein nature of these sites was clearly indicated by their sensitivity to trypsin degradation. Likewise, lipid crude extracts from Solanaceae were able to compete with [^{3}H]17β-estradiol for binding to the estrogen receptor (ER) from breast cancer MCF-7 cells as well as with estrogen binding sites present in this plant species [5,6,12].

Skeletal muscle was considered a non-classical estrogen target for a long time, though little is known about the effect of estrogens on this tissue. However, in recent years, both ERs have been found to be present in mouse, human, and pig skeletal muscle as well as in myoblasts from rat and mouse [13-16]. It is now known that estrogens exert actions in skeletal muscle. For example, muscle degenerative pathologies like sarcopenia, that is observed in menopausic women, are related to decreased levels of estrogens [17]. Also, estrogens have been shown to be an important factor in the protection of muscle from exercise-induced muscle damage [18]. Thus, the presence of ERs in skeletal muscle tissue and the capacity of phytoestrogens to bind to estrogen receptors, make this tissue a target for phytoestrogens actions.

Adult skeletal muscle increases its size and shows a remarkable capacity to adapt to trauma and injury. However, skeletal muscle cells are postmitotic and cannot replicate. Therefore, any increase in myonuclear number required for growth or repair of damaged muscle depends on satellite cells, a pool of myogenic precursor cells. This distinct population of mononucleated [19,20] was first described by Mauro (1961) [21]. They owe their name to their localization under the basement membrane but outside the plasma membrane of the muscle fiber. Their colocalization with blood vessels [22] places satellite cells in an optimal position to respond to intrinsic signals from both the skeletal muscle fiber itself and from changes in the systemic environment. Satellite cells exist in a quiescent state after birth and begin to proliferate in response to regulatory factors during development and in cases of muscle injury [23-25]. Then, satellite cells are activated in response to both physiological stimuli, such as exercise, and to pathological conditions, such as injury and degenerative diseases. During development and regeneration, quiescent satellite cells are activated and start to proliferate. At this stage, they are often referred to as myogenic precursor cells or myoblasts [26]. The interest for us, is the presence of estrogen receptors (ERs) in satellite cells [14]. In addition, we demonstrated that E2 abrogates the apoptosis induced by oxidative stress in myoblasts through ERs [27]. Furthermore, during steroid-induced muscle growth, the hormone induces activation of muscle satellite cells [28]. Thus, this cells group that is relevant for the muscle physiology, could be affected by phytoestrogens.

Apoptosis can be triggered through the mitochondrial pathway or the death receptor-mediated pathway, both leading to caspase activation that ultimately results in nucleus condensation and DNA fragmentation [29,30]. As mentioned above, plant extracts exert many pharmacological functions, including apoptosis in tumor cells [31-33]. These apoptotic effects have been attributed to the presence of different compounds like phytosterols in the plant lipid extracts preferentially [34-36].

It is known that Solanaceae family provide significant amount of phytoestrogens [2,37]. Previously, we evidenced the presence of molecules functionally and structurally analogous to E2 in lipid extracts of the Solanaceaes [6].

The goal of the present study was to investigate the effects of lipid extracts from *N. glauca* on myoblastos, in relation to apoptosis; since apoptosis is a possible cause of diminution of satellite cells number in sarcopenia or other myopathies [38]. In addition, our aim was to contribute to the

knowledge about the chemical structure of the phytoestrogens present in the lipid fraction of *N. glauca*, responsible of the effects observed on C2C12 cell line.

2. Material and Methods

2.1. Materials

Anti-phospho-Akt (Ser473) and anti-HSP27 antibodies were from Cell Signaling Technology Inc (Danvers, MA, USA).

Anti-beta tubulin (1:10000) antibody was obtained from Thermo Fisher Scientific, Inc (Rockford, IL, USA). DAPI and MitoTracker Red (MitoTracker Red CMXRos) dyes were from Molecular Probes (Eugene, OR, USA). The ECL blot detection kit was provided by Perkin-Elmer, Inc (Waltham, MA, USA). The protein molecular weight marker was from Amersham (Buckinghamshire, England). TUNEL assay kit was from Promega (Promega Corp., Madison, WI), Cell Event Caspase 3/7 detection reagent was from Invitrogen (Carlsbad,CA). All the other reagents used were of analytical grade.

2.2. Plant Material

Nicotiana glauca plant specimens were collected from their natural habitats in Buenos Aires Province, Argentina and were grown under green-house conditions.

2.3. Extraction of lipid extracts

The starting material plant was obtained from dried leaves of *N. glauca* (126 g), which was extracted for two times with 96% ethanol (3 L) at room temperature for 10 days. The ethanolic extract (crude extract) was concentrated under reduced pressure giving 26.3 g (21%). The residue was successively partitioned with hexane, chloroform and ethyl acetate. The extracted solutions were evaporated under reduced pressure and then lyophilized to yield 993 mg (3.8%) of hexane sub-extract, 61 mg (0.23%) of chloroform sub-extract and 164 mg (0.62%) ethyl acetate sub-extract. 25 mg of final residue of each phase was solubilized in isopropanol (5 ml) and was stored at -20°C.

2.4. Phytochemical screening

Phytochemical examinations were carried out for all the sub-extracts as per the standard methods [39]. For the detection of alkaloids was used the Dragendroff's test. The flavonoids were identified through the Fast Blue Salt reagent. The anthraquinones were detected using the Bornträger´s test, and triterpenes and steroids were recognized using Libermann Burchard's test.

2.5. Cell culture and treatment

C2C12 murine skeletal myoblasts obtained from American Type Culture Collection (Manassas, VA, USA) were cultured in growth medium (DMEM) supplemented with 10% heat-inactivated (30 min, 56 8C) fetal bovine serum, 1% nistatine, and 2% streptomycin. These highly myogenic cells have been widely used to study muscle functions [27,40,41]. The C2C12 cell line are murine myoblasts derived from satellite cells, whose behavior corresponds to that of progenitor lineage. This cell line is a subclone of C2 myoblasts [42] which spontaneously proliferate, differentiate and synthesize characteristic muscle proteins in culture [43,44]. Since C2C12 cells are comparable to satellite cells in muscle fibers [45], they represent an appropriate experimental model of them.

Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air. Cultures were passaged every 2 days with fresh medium. The treatments were performed with 70–80% confluent cultures (120 000 cells/cm^2) in medium without serum for the time indicated in specific experiments. During this preincubation, cells were exposed to the crude extract from *N. glauca* (1:1000 dilution of the lipid extract in DMEM without serum), each sub-extract: hexane, chloroform and ethyl acetate or vehicle [0.001% isopropanol (control)]; were added 20 min before induction of apoptosis with hydrogen

peroxide (H_2O_2) 0.5 mM at the times indicated (ranging from 30 min to 3 h). The time and concentration range of the oxidant has been previously used to study apoptosis in C2C12 cells [27,41,46]. H_2O_2 was diluted in culture medium without serum at a final concentration of 0.5 mM in each assay. After treatments, cells were lysed using a buffer composed of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.2 mM Na_2VO_4, 2 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP40, 20 mg/ml leupeptin, and 20 mg/ml aprotinin. Protein concentration was estimated by the method of Bradford (1976) [47], using BSA as standard. For microscopical assays, cells were cultured in chamber slides.

2.6. Terminal Transferase dUTP Nick End Labeling (TUNEL) assays

After the specific treatments, cells grown over coverslips, were processed for *in situ* localization of nuclei exhibiting DNA fragmentation by the technique of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin nick-end labeling (TUNEL) with the use of the apoptosis detection kit DeadEndTM Fluorometric TUNEL System (Promega , Madison, WI). The protocols were followed according to the manufacturer´s instructions. Then cells were mounted with 95% glycerol and analyzed by conventional fluorescence microscope (NIKON Eclipse Ti-S equipped with standard filter sets to capture fluorescent signals, and images were collected using a digital camera). At least 500 cells of each experimental condition were counted and apoptotic cells were identified by nuclei staining (TUNEL-positive cells). The results were expressed as percentage of apoptotic nuclei.

2.7. Quantitation of apoptotic cells

After treatments, cells were fixed with methanol at -20 °C for 30 min and then washed with PBS. Fixed cells were incubated for 30 min at room temperature in darkness with 1:500 of a stock solution of DAPI (5 mg/ml) and next washed with PBS. Cells were mounted on glass slides and examined using a fluorescence microscope (NIKON Eclipse Ti-S) equipped with standard filter sets to capture fluorescent signals. Images were collected using a digital camera. Apoptotic cells were identified by the condensation and/or fragmentation of their nuclei. The results were expressed as percentage of apoptotic cells. A minimum of 500 cells was counted for each treatment from at least three independent experiments.

2.8. MitoTracker red staining

Coverslips with adherent cells were stained with MitoTracker red (Molecular Probes), which was prepared in dimethyl sulfoxide and then added to the cell culture medium at a final concentration of 1 mmol/l. After 15 to 30 min incubation at 37 °C, cells were washed with PBS and fixed with methanol at -20 °C for 30 min. Finally, the coverslips were analyzed by conventional fluorescence microscopy as described previously.

2.9. Caspase-3/7 Activity Assay

After specific treatments, cells were labeled with 6 μM CellEventTM caspase-3/7 green detection reagent in PBS with calcium and magnesium for 30 min at 37 °C in the dark. Finally, the stained cells were analyzed with a conventional fluorescence microscope (NIKON Eclipse Ti-S equipped with standard filter sets to capture fluorescent signals, and images were collected using a digital camera). At least 500 cells of each experimental condition were counted and activation of caspases were identified by green fluorescence in nuclei (Caspase-3/7-positive cells). The results were expressed as percentage of Caspase-3/7-positive cells.

2.10. Western blot analysis

Cell cultures were scrapped and resuspended using a 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 phenylmethylsulfonyl fluoride (PMSF), 20 mg/ml leupeptin, and 20 mg/ml aprotinin). Lysates were collected by aspiration and

centrifuged at 12,000 g for 15 min. The protein content of the supernatant was quantified by the Bradford procedure [47] using BSA as standard. Then, lysate proteins dissolved in Laemmli [48] sample buffer were separated on 10-12% SDS polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. Relative migration of unknown proteins was determined by comparison with molecular weight colored markers (Amersham, Piscataway, NJ, USA). Membranes were blocked 1 h at room temperature in PBS–T buffer (PBS 0.1% Tween-20) containing 5% dry milk. Membranes were incubated with different primary antibodies overnight at 4 °C, then washed three times in PBS–T and incubated in PBS–T containing 1% dry milk with peroxidase conjugated secondary antibodies for 1 h at room temperature. Next, membranes were visualized using an enhanced chemiluminescent technique according to the manufacturer's instructions. For reprobing with other antibodies, membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 50 mM mercaptoethanol) for 30 min at 55 °C, washed for 10 min in PBS–T, and then blocked and blotted as described above.

2.11. Statistical analysis

Data analysis was performed using standard statistical packages (InfoStat System, Córdoba, Argentina [49]. All values are shown as the mean ± S.D. of at least three independent experiments. The data were considered statistical significant when $P<0.05$.

3. Results

3.1. Crude extract from Nicotiana glauca induces apoptosis in skeletal muscle cells

In previous work, we observed that E2 abrogates H_2O_2-induced apoptosis of C2C12 myogenic cells [27]. Now we investigate whether the lipid extracts from *N. glauca* also act as the hormone. C2C12 cells were challenged with crude extract during the times indicated and apoptotic events were investigated (Methods). The nuclear dye DAPI showed morphological changes typical of apoptosis such as nuclear fragmentation/condensation (pyknotic nuclei) after treatment with the crude extract from *N. glauca*, which represented near to 70% of the cultured muscle cells in similar fashion as those treated with H_2O_2 0.5 mM (data not shown). In addition, morphological changes and cellular redistribution of mitochondria could be detected in C2C12 cells treated with ethanolic extract from *N. glauca* and then stained with the fluorescent mitochondrial probe MitoTracker red (Methods) as described above. Thus, Figure 1 shows that cells treated with vehicle (control) display 'spiderweb' or uniform distribution of mitochondria through the cytosol. On the other hand, when apoptosis was induced with H_2O_2 or the cells were treated with the crude extract, we observed reduced mitochondria size, 'pyknotic', and characteristic clustering of the organelles around the nucleus (which represented near to 70% of the cultured muscle cells), events associated to apoptosis. To confirm those observations, we evaluated the effects of the crude extract from *N. glauca* on skeletal muscle cells by TUNEL assays (Figure 2). We perform the same experimental conditions as before. Cells treated with the crude extract exhibited a large increase in DNA fragmentation (63 % ± 9.09 of TUNEL positive cells above the control), similar to the values obtained with H_2O_2 treatment (Figure 2).

(**a**) (**b**) (**c**)

Figure 1. Crude extract from *N. glauca* induce changes in morphology and localization of mitochondria in C2C12 muscle cells. C2C12 cells grown on coverslips as 60–70% confluent monolayers were treated (see below), stained with MitoTracker Red, and fixed with methanol as described under Materials and Methods Section. (**a**) Untreated cells. (**b**) Cells treated with crude extract (1:1000 dilution) during 2 h. (**c**) Cells treated with H_2O_2 0.5 mM during 2 h. Untreated cells present normal mitochondrial morphology and distribution throughout the entire cell distant to the nucleus or display 'spiderweb' mitochondria; but cells treated with the crude extract exhibit mitochondria clustered around the nucleus with condensed or pyknotic aspect as cells treated with H_2O_2. At least ten fields per slide and three independent cultures were examined. Representative photographs are shown. Magnification: 63X.

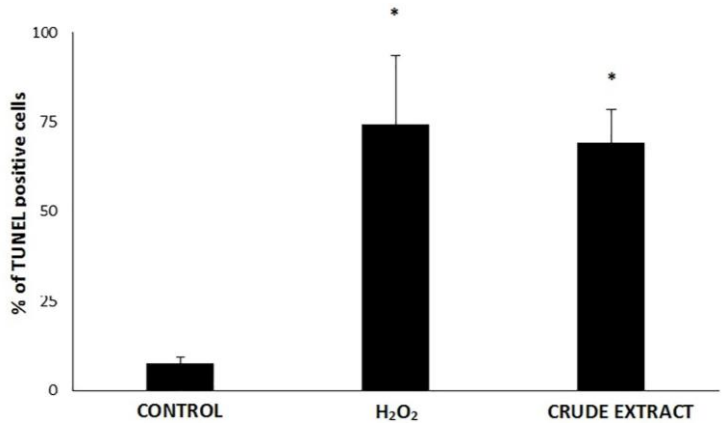

Figure 2. Crude extract from *N. glauca* induces apoptosis in C2C12 cells. C2C12 cells untreated (CONTROL) or incubated with crude extract from *N. glauca* for 1 h (CRUDE EXTRACT) or with the apoptotic inducer H_2O_2 (0.5 mM, 2 h). Then, apoptosis was determined by TUNEL assays as described under Materials and Methods Section and expressed as the percentage of TUNEL positive cells in the coverslips. Each value represents the mean of three independent determinations ±SD; *P <0.05 with respect to the control.

3.2. Crude extract from Nicotiana glauca induced Apoptosis involves Caspase-3/7

Within the caspase family, the effector caspases-3 and -7, orchestrate the destruction phase of apoptosis that results in the controlled dismantling of a range of key proteins within the cell and its subsequent disposal [50]. Moreover, one of the most evident and specific features of apoptosis is the degradation of the DNA into numerous fragments, driven by the activation of caspase-3 [51], the central effector caspase, which makes it an attractive biomarker of apoptosis. To address whether the apoptotic action of the crude extract from *N. glauca* on C2C12 muscle cells is exerted through caspases 3/7 activation, C2C12 cells were treated with ethanolic extract as before (Methods) and analyzed by fluorescent microscopy using the specific Cell Event Caspase 3/7 detection probe. As shown in Figure 3, the crude extract induces the caspases 3/7 activation (86% ± 7.21 of caspase positive cells above the control) in similar fashion as hydrogen peroxide.

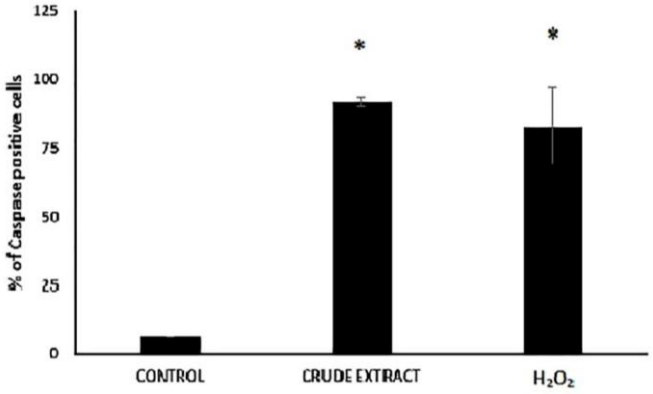

Figure 3. Crude extract from *N. glauca* induces Caspase 3/7 activation. C2C12 cells grown on coverslips as 60–70% confluent monolayers were treated with the indicated stimuli as before and were analyzed for caspase activation using Cell Event Caspase 3/7 detection probe (Methods). (CONTROL) untreated cells, (CRUDE EXTRACT) cells incubated with crude extract from *N. glauca* for 1 h to 2h, (H_2O_2) cells treated with H_2O_2 (0.5 mM, 1 h to 2h). Experiments were repeated at least three times with essentially identical results. ±SD; *P <0.05 with respect to the control.

3.3. Effect of hexane, chloroform and ethyl acetate sub-extracts from Nicotiana glauca

With the aim to characterize the chemical structure of the apoptotic effectors present in the crude extract of *N. glauca*, extraction procedure with solvents was carried out (Methods). Thus, crude extract of *N. glauca* was partitioned with hexane, chloroform and ethyl acetate (Methods) and each sub-extract was tested for apoptotic activity in skeletal muscle cells by TUNEL assays and caspases activation. Regards caspase activation, the assay indicated that hexane and to a lesser extent chloroform sub-extracts (90 % ± 4.6 and 55% ± 14 of caspase positive cells above the control, respectively) induce apoptosis activating caspases 3/7 (Figure 4). The same results were obtained, evaluating the caspase activation through flow cytometry (data not shown). Congruent with this data, TUNEL assays shown that hexane and to a lesser extent chloroform and ethyl acetate sub-extracts are able to induce apoptosis (98% ± 1.01, 67% ± 18.38, 44.6% ± 12.7 TUNEL positive cells respectively) (Figure 4).

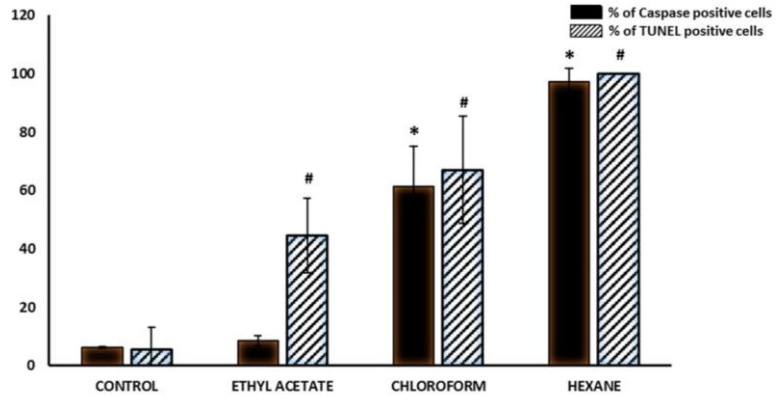

Figure 4. Hexane, chloroform and ethyl acetate sub-extracts from *N. glauca* induce apoptosis through caspase 3/7 in C2C12 cells. C2C12 cells were treated with the indicated stimuli (ETHYL ACETATE, CHLOROFORM or HEXANE) as before. CONTROL, untreated cells. Then, apoptosis was determined by TUNEL assays as described under Materials and Methods Section and expressed as the percentage of TUNEL positive cells in the coverslips. Likewise, other cultures were treated with sub-extracts as before and were analyzed for caspase activation using Cell Event Caspase 3/7 detection probe (Methods). Each value represents the mean of three independent determinations ±SD; *P <0.05 with respect to the control.

3.4. Lipid extracts obtained from Nicotiana glauca activate stress signaling

Against a potentially damaging stresses the cell activates its defense mechanism/survival signaling pathways, as a first and rapid response that allows to tolerate and/or to recover from the damage imposed. As injury continues, such mechanisms are no longer sufficient. Thus, when the injurious signal persists and exceeds the mechanisms of survival, the net effect is the cell death. Among the cellular responses to stress, here we evaluated HSP27 and Akt phosphorylation levels in response to the lipid sub-extracts obtained from *N. glauca* crude extract. C2C12 cell cultures were incubated with each sub-extract during 30 min followed by measurement of HSP27 and Akt phosphorylation by immunoblot analysis of cell lysates. As shown in Figure 5, Western blot analysis using anti-phospho-Akt and anti-phospho-HSP27 antibodies revealed Akt and HSP27 activation (phosphorylation) in response to hexane, chloroform and ethyl acetate sub-extracts treatments. Immunocytochemistry studies using fluorescent microscopy and the same antibodies were congruent with the Western blot results, Figure 6.

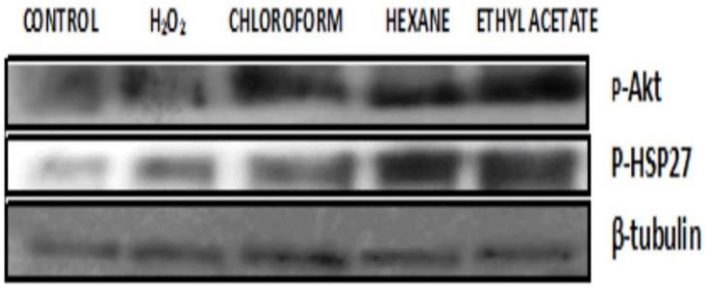

Figure 5. Hexane, chloroform and ethyl acetate sub-extracts from crude extract from *N. glauca* trigger Akt and HSP27 phosphorylation. C2C12 cells were treated with the indicated stimuli (CHLOROFORM, HEXANE, ETHYL ACETATE or H_2O_2) as before. CONTROL, untreated cells. Cell lysate proteins from each condition containing equivalent protein amounts (25 mg) were fractionated by SDS-PAGE, transferred to PVDF membranes, and western blotted with Phospho-Akt or Phospho-HSP27 antibodies as described in Materials and Methods Section. B-tubulin levels are shown as protein loading control. Immunoblots representative are shown.

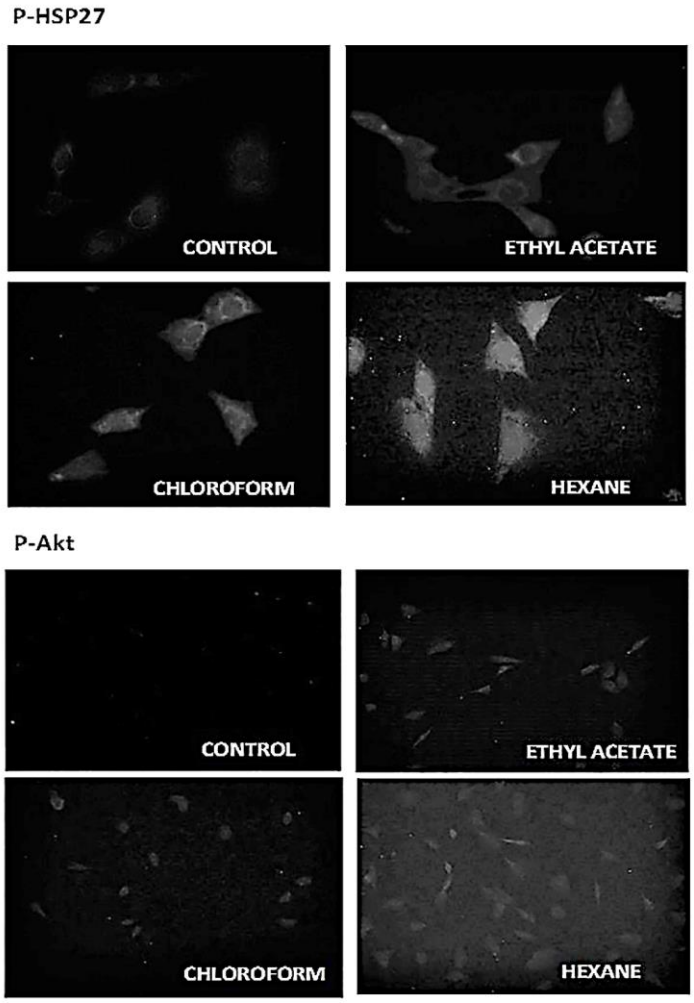

Figure 6. Hexane, chloroform and ethyl acetate sub-extracts from crude extract from *N. glauca* trigger Akt and HSP27 phosphorylation. C2C12 cells were treated with the indicated stimuli (CHLOROFORM, HEXANE, ETHYL ACETATE or H_2O_2) as before. CONTROL, untreated cells. Fluorescence microscopy of p-HSP27 and p-Akt phosphorylation. HSP27 or Akt phosphorylated (green fluorescence) were stained by using anti- phospho-HSP27 or anti- phospho-Akt primary antibody, respectively and Alexa 488-conjugated secondary antibody. Experiments were repeated at least three times with essentially identical results (Magnification for P-Akt: 20x and for P-HSP27: 63x).

4. Discussion

As was described in Introduction, Solanaceae family provide significant amount of phytoestrogens [37]. Phytoestrogens are supplements and widely marketed as a natural alternative to estrogen replacement therapy or to treat a wide range of health conditions. The risk of adverse effects of its use, however, has not been fully studied. Moreover, the molecular mechanism of the side effects is unknown for much of them.

In this work, the murine skeletal muscle cells were treated with a crude ethanol extract from *N. glauca,* with the lipid sub-extracts obtained from that or with H_2O_2 as a positive control of apoptosis. As a first approach we evaluated the effects of those treatments seeing the cellular morphology using DAPI and mitotracker dyes. Cells exposed to the crude extract, to the hexane or to the chloroform sub-extracts shown typical apoptotic morphology (nuclear condensation / fragmentation, mitochondrial picnosis and clustering of the organelle near the nucleus) similar to H_2O_2 treatment. Opposing to ours previous works in which we observed that 17β-estradiol protects the skeletal muscle cells inhibiting the H_2O_2- induced apoptosis [52], here we found that the crude

extract and sub-extracts of hexane and chloroform from *N. glauca* induced apoptosis in C2C12 cells. Indeed, in accordance with the results from Tunel assays, we found an important activation of caspases when cells were treated with the lipid hexane and chloroform sub-extracts, being the hexane sub-extract the most potent. On the other hand, the ethyl acetate sub-extract was unable to induce higher levels of apoptosis, throwing values similar to controls. These results suggest that molecules of lipidic nature, concentrated preferentially in the hexane and in less part, the chloroform sub-extracts, are responsible for the apoptotic stimuli.

We have also observed that short treatments times (30 min) with the sub-extracts from *N. glauca*, induced the activation of a rapid cellular defense response with Akt and HSP27 activation as it was observed in previous works with H_2O_2 as the apoptotic inductor [52] . Indicating, that cells sense an injury from those treatments. However, when we perform longer treatments (1 to 2h) (data not shown), cells were unable to sustain those survival response and turn into apoptosis.

Finally, in this work, we were interested to beginning with the identification of the molecular actors during the apoptotic signaling induced by the lipid extracts. For the first approaches we analyze caspase activity. In this assay the substrate (amino acid peptide DEVD) used to evaluate the caspase activation is recognized by both caspases 3 and 7. In view of this, our results suggest that the compounds from *N. glauca* trigger apoptosis involving caspase pathways, but we are unable by this method to identify the specific caspase involved.

These results, opposite to those observed by us with E2, are in agreement with the different behaviors of phytoestrogens due their diverse ways to bind the ERs. Indeed, they can act like partial ER agonists or antagonists [53]. The differential effects of agonists and antagonists on receptor activity in a given cell context have been ascribed to different conformations of the receptor ligand complex, as well as by differences in the interaction with transcriptional coactivator and corepressor proteins and other transcription factors. In addition, to increase the complexity of molecular mechanisms that mediate the phytoestrogens effects, it have been demonstrated that these compounds have additional cellular activities not ascribed to activation of the ERs, such as regulation of cell-signaling pathways [53,54].

With the aim to obtain some knowledge about the chemical structure of the compounds responsible of the effects here observed on satellite cells, we performed phytochemical screening of the sub-extracts from *N. glauca*. We observed the presence of flavonoids, anthraquinones, triterpenes and steroids in the sub-extracts of hexane and chloroform. The presence of alkaloids in these sub-extracts was not observed. In the ethyl acetate sub-extract the presence of all the chemical structures examined was detected, but probably, are present in a low concentration respect the others sub-fractions. An exciting prospect of the future research orientated to identify the compounds of hexane and chloroform sub-extracts lies in comprehension of their properties and complete determination of benefit/risk ratio.

The data reported here, on the effects of lipid extracts from *N. glauca* on myoblasts suggest that a possible use of this medicinal plant could affect the skeletal muscle homeostasis. Since, muscle satellite cells are critical for successful muscle regeneration and repair [55]. Thus, a possible compound to use in hormone replacement therapy to overcome menopausal symptoms like osteoporosis, could affect skeletal muscle leading to sarcopenia. Increasing the ageing symptoms. Clearly, additional studies are then necessary to further elucidate the signaling mechanisms, which mediate the apoptotic action of compounds from *N. glauca* in skeletal muscle cells. This knowledge may be of relevance to develop strategies for avoid undesirable side effects increasing the potential as natural pharmaceutical compound of phytoestrogens.

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Author Contributions: L.M., M.B.F. and A.V. conceived and designed the experiments; D.L. and F.A.M. performed the experiments; L.M. maintain the cellular cultures; L.M., M.B.F. and A.V. interpreted the results, contributed reagents/materials/analysis tools and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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