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Original article

Celastrol and Rhynchophylline in the mitigation of simulated muscle atrophy under *in vitro*

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ABSTRACT

Background: Muscular atrophy (MA) is a disease of various origins, i.e., genetic or the most common, caused by mechanical injury. So far, there is no universal therapeutic model because this disease is often progressive with numerous manifested symptoms. Moreover, there is no safe and low-risk therapy dedicated to muscle atrophy. For this reason, our research focuses on finding an alternative method using natural compounds to treat MA. This study proposes implementing natural substances such as celastrol and Rhynchophylline on the cellular level, using a simulated and controlled atrophy process. *Methods:* Celastrol and Rhynchophylline were used as natural compounds against simulated atrophy in C2C12 cells. Skeletal muscle C2C12 cells were stimulated for the differentiation process. Atrophic conditions were obtained by the exposure to the low concertation of doxorubicin and validated by FoxO3 and MAFbx. The protective and regenerative effect of drugs on cell proliferation was determined by the MTT assay and MT-CO1, VDAC1, and prohibitin expression. *Results:* The obtained results revealed that both natural substances reduced atrophic symptoms. Rhynchophylline and celastrol attenuated atrophic cells in the viability studies, morphology analysis by diameter measurements, modulated prohibitin VDAC, and MT-CO1 expression. *Conclusions:* The obtained results revealed that celastrol and Rhynchophylline could be effectively used as a supportive treatment in atrophy-related disorders. Thus, natural drugs seem promising for muscle regeneration.

1. Introduction

Muscle atrophy can be caused by various reasons, such as lack of mechanical stimulation, starvation, cancer, genetic diseases, or even presence in a vestigial or no gravity environment. The exciting and reasonable solution to mentioned dysfunction would be the use of pharmacological agents to inhibit the atrophy of muscle tissue, even under conditions that could induce and stimulate the occurrence of muscular atrophy. Muscle atrophy can occur during long periods of inactivity. This "inactivity phenomenon" can be caused by poor nutrition (as a result of cancer, celiac disease, or irritable bowel syndrome), age, or genetic disorders, e.g., spinal muscular atrophy (SMA) (Prior et al., 1993). Other diseases and chronic conditions can induce atrophy, e.g., amyotrophic lateral sclerosis, arthritis, and myositis. The most difficult to treat are atro-

Abbreviations: Akt1, serine/threonine-protein kinase or AKT kinase 1; CLS, celastrol; DEX, dexamethasone; DOX, doxorubicin; ERK1/2, extracellular signal-regulated kinase 1/2; HSF1, Heat shock factor 1; HSP, heat shock protein; ICC, immunocytochemistry; MA, Muscular atrophy; MAFbx, Muscle Atrophy F-box gene; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; MTCO1, Mitochondrially Encoded Cytochrome *C* Oxidase I; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MuRF1, Muscle RING Finger protein-1; NLS, nuclear localization signals; RA, rheumatoid arthritis; Ryn, Rhynchophylline; SMA, spinal muscular atrophy; VDAC, voltage-dependent anion channel.

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phies caused by genetic disorders, and currently, available approaches are ineffective and expensive (Jędrzejowska and Kostera-Pruszczyk, 2020). Muscle mass weight depends primarily on the ratio of processes and proteins that affect healthy muscle cell proliferation to those that affect their atrophic development. During muscle tension absences or relaxation - the process of muscle tissue protein degradation is controlled by the transcription factor FoxO, directly related to the ubiquitin-proteasomal system (Arun et al., 2017). In this case, a reduced actin activity stimulates the FoxO factor, which stimulates muscle-specific ligases such as MaFbx (Muscle atrophy F-box) MuRF1 (Muscle RING Finger 1) - crucial in stimulation and responsibility for the pathophysiological process. These processes can also be stimulated by the transcriptional activity of glucocorticoid receptors, which depends on external factors like adrenal hormones such as DEX (dexamethasone) (Gwag et al., 2013).

Currently, there is no golden solution for the regeneration of the atrophic muscles. Therapies based on natural substances can support the available therapeutic protocols and be more safe and more beneficial for the patients. Recently, a beneficial role of herbal medicine against muscle atrophy was demonstrated (Li et al., 2021), e.g., of *valeriana fauriei* (Kim et al., 2022) or *Abelmoschus manihot L. Medik* (Anggi, 2020). In this study, we aimed to validate celastrol and Rhynchophylline for this purpose. Celastrol is a pentacyclic triterpenoid (belonging to the quinone structured compounds) naturally occurring in the roots of the plant *Tripterygium wilfordii* (Thunder duke vine), used in traditional Chinese medicine. This compound is of high interest due to its potential use in the treatment of obesity, diabetes, and cardiovascular diseases caused by poor nutrition (mostly diets containing highly processed and sweetened products) - which was confirmed by researchers from centers in the USA, China, and Korea studies in the years 2011–2019 (Wastag et al., 2020)(Moreira et al., 2018). The feasible therapeutic mechanism of this compound concerns the induction of the HSF1 protein (encoded by the HSF1 gene), which affects the HSP70, HSP90 heat shock proteins proliferation and regulation of what is commensurate with the activity of the PGC-1a transcription coactivator complex. The PGC-1a protein (encoded by the PPARGC1A gene) plays a crucial role in energy metabolism regulation due to its effect on mitochondrial activity and biogenesis, which is crucial in muscle functioning (Gwag et al., 2013)(Gwag et al., 2015). Rhynchophylline (Ryn) is an alkaloid derived extracted from of *Uncaria rhynchophylla* (Ballester Roig et al., 2021)(Kaneko et al., 2020). There was noted that Ryn enhances cellular proliferation, differentiation, neuroprotection (He et al., 2014)(Dey and Mukherjee, 2018) (Akkol et al., 2021), and also acts as an antioxidant (Kaneko et al., 2020) (Ravipati et al., 2014), and as an anti-inflammation compound (Dey and Mukherjee, 2018). Thus the broad spectrum of applications of celastrol and Rhynchophylline place them as good candidates for potential usage in neurological diseases (Akkol et al., 2021) and validation in muscle-related disorders. Natural compounds seem safe and might be combined with currently used conventional methods.

This study evaluates if natural compounds can improve muscle cells' recovery from the atrophic state. An *in vitro* model of muscle atrophy based on C212 cells was used in the study. Natural compounds, i.e., celastrol and Rhynchophylline were implemented to overcome atrophic symptoms. Simulated atrophy and its neutralization were detected by specific markers FOXO3 or MAFbx. The effect of the treatment with natural compounds was determined by the viability assay, MTCO-1, VDAC, and prohibitin visualization. The obtained results can contribute to muscle atrophy co-treatment by using natural drugs.

2. Materials and methods

2.1. Cell culture

C2C12-normal mouse myoblasts (American Type Culture Collection, ATCC®, LGC Standards Sp. z o.o., Poland) were used in the study. C2C12 cells were grown in DMEM (Sigma-Aldrich, Poznan, Poland), low glucose (1 g/L), and GlutaMAX™ Supplement (Gibco). The culture medium was supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), and penicillin/streptomycin (100 U/mL, Gibco). Cells were passaged twice a week. The cells were grown in an incubator at 37 °C with 5% CO_2. Cells were tested routinely for mycoplasma once per month.

2.2. Cells' differentiation induction

Cells' differentiation process was initiated at 80% confluency of cells. The standard culture medium for C2C12 was replaced by DMEM (Sigma-Aldrich, Poznan, Poland) of high glucose (4.5 g/L), and with 2% horse serum (HS, Gibco™ Horse Serum, heat-inactivated). The culture medium was changed every second day. Myotubes Cells were differentiated for five days before experiments. Differentiated control cells are shown in Fig. 1 SM as CTRL.

2.3. Natural compounds preparation

Celastrol (C0869) and Rhynchophylline (PHL80381) presented in Fig. 1 were obtained from Sigma-Aldrich (Poland, Poznan) and used for the study to counterbalance cells from the atrophic conditions. Stock solutions of 10 mM concentration were prepared in MilliQ water and were used to prepare dilutions in the range from 10 nM to 200 μM (final dilutions in cells' culture media), which enabled the selection of the therapeutic drugs' concentration used in the study.

2.4. Doxorubicin exposure for atrophy stimulation

When cells differentiated into myotubes after 5 days, atrophic conditions were provoked by various concentrations (1–20 μM) of doxorubicin (DOX) post 24 h exposure time. Doxorubicin final dilutions were prepared freshly in a cell culture medium. The procedure was based on the experimental literature data (Willis et al., 2019)(Burke et al., 2020). In this study, we have selected low cytotoxic DOX concentrations 2 and 4 μM for the simulation of the atrophic conditions (Fig. 1-SM). The atrophic state was validated by microscopic observations of myoblasts differentiation inhibition and differentiation marker (FoxO3) evaluation.

2.5. Cell differentiation examination by immunocytochemical (ICC) staining of forkhead box O3 (FoxO3)

C2C12 cells were seeded on 10-well microscopic slides (Equimed, Poland) and differentiated 5 days according to the *2.2* protocol described above. Then atrophy was induced in C2C12 cells by 2 and 4 μM DOX for 24 h. Cells were washed in PBS (BioShop, Poland) before and after fixation in 4% formaldehyde (Roth, Germany). Phospho-FOXO3A (Ser253) polyclonal antibody (cat no. PA5-104701, ThermoFisher) in 1:500 dilution was used for the immunocytochemical reaction. The immunocytochemical (ICC) assay was performed according to our previous study (Novickij et al., 2020). Peroxidase-ABC labeling assay (Abcam, United States; Cat. no ab80a36) was used for the samples. Cells on the microscopic slides were incubated with a diaminobenzidine-H_2O_2 mixture to visualize the peroxidase label and counterstained with

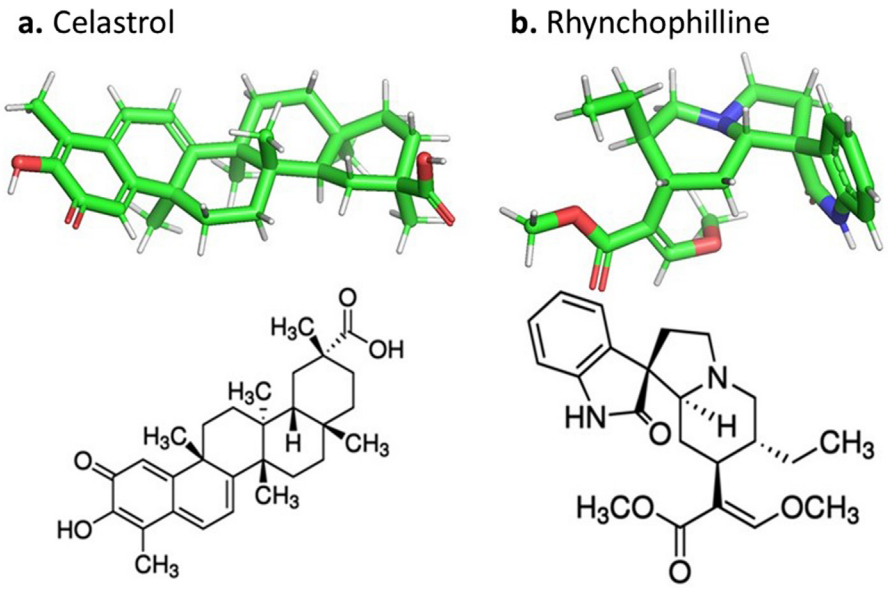

Fig. 1. Structural representation of (**a**) celastrol, and (**b**) Rhynchophylline (Compounds' structures were prepared and their geometry optimized in Avogadro software, visualizations were created in Pymol software). Simple structures originate from https://www.sigmaaldrich.com/.

hematoxylin (Roth, Germany) for 60 sec. The samples were analyzed with an upright microscope (Olympus BX51, Japan). Immunostained cells on microscopic slides were determined by counting 100 cells in randomly selected regions. The results were positive if the stained reaction was observed in more than 5% of cells. The intensity of immunocytochemical (ICC) staining was evaluated as (-) negative, (+) weak, (++) moderate, and (+++) strong.

2.6. Cytotoxicity assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for the drugs' cytotoxicity. All drugs' dilutions were performed in the cultivation media. Cells were detached by trypsinization (0.25% trypsin-EDTA, Sigma), and seeded in density $2x10^4$ on 96-well plates (Nunc). Cells were cultivated overnight to adhere and afterward incubated with various concentrations of celastrol, Rhynchophylline, or doxorubicin for 24 h. The control cells, corresponding to 100% of the mitochondrial activity, were incubated in a culture medium (DMEM). For the final viability measurements, the medium was replaced with 100 μL per well of 5 mg/mL (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) diluted in phosphate-buffered saline (PBS, Bioshop, Poland). The MTT experiments were performed according to the manufacturer's procedure. The absorbance was measured in the multiplate reader (GloMax Promega) at a wavelength of 570 nm. The results were presented as a percentage of control untreated cells. Each experiment was performed in a minimum of eight repetitions. Results were presented as mean values with standard deviations. IC50 was calculated using the analysis in GraphPad Prism software 7.0, where nonlinear regression is used for analysis (https://www.graphpad.com/support/faq/how-to-determine-an-icsub50sub/).

2.7. Myoblasts diameter measurements

Cells were seeded in the density of $4x10^4$ on 6-well plates, coated with collagen I (Gibco, Life Technologies, Poland), and differentiated for 5 days as was described previously (*section 2.2*) (Fig. 1SM.). Then atrophic conditions were stimulated in C2C12 cells by 4 μM DOX for 24 h. Next, cells were washed by a cell culture medium, and celastrol or Rhynchophylline was added in various concentrations for the next 24 h., A minimum of three repetitions were performed. Then microscopic observations were performed, and the obtained images were analyzed by ImageJ 1.53 k free software (Schindelin et al., 2012). A color threshold was set as black and white to simplify images; the scale was set in the software to be compatible with the microscopic visualization, and the width and length of cells were measured.

2.8. Combination therapy – atrophy mitigation

Firstly, cells were seeded in the density of $4x10^4$ on 12-well plates (Greiner, Germany), and differentiated for 5 days as was described previously (*section 2.2*). Then atrophic conditions were stimulated in C2C12 cells by 4 μM DOX for 24 h. After this time, cells were washed by a cell culture medium, and celastrol or Rhynchophylline was added in to various concentrations for the next 24 h. Then cells were dedicated to further analysis, i.e., viability and immuno- assays.

2.9. Immunofluorescent reactivity of muscle atrophy F-box gene (MAFbx)

Cells were seeded on cover microscopic slides (Menzel, Germany) and differentiated for 5 days. After atrophy simulation by 4 μM DOX, cells were washed in PBS (BioShop, Poland) and fixed in 4% formaldehyde (Roth, Germany). Then the cells were washed 3x in PBS, and incubated for 5 min with PBS with 1% Triton 100X, blocked for 60 min with 4% FBS. MAFbx Antibody (F-9) conjugated with Alexa Fluor® 488 (1:200, sc-166806 AF488, Santa Cruz) was diluted in PBS with 0.5% FBS and added for 60 min at 37 °C and 5% CO_2. After washing in PBS, cells were mounted with DAPI (nuclei marker) Mounting Medium (Roth, Germany). The samples were analyzed by a confocal laser microscope (Olympus FluoViewer 1000, Japan). Fiji package of ImageJ 1.53 k software (ROI Manager, Multi Measure) (Schindelin et al., 2012) was used to quantify the mean fluorescent signal.

2.10. Immunocytochemical staining of MT-CO1, VDAC1, prohibitin

Cells were seeded on 3-well microscopic slides (Roth, Germany) and differentiated for 5 days. Large wells of the microscopic slides were selected in particular for the cells' differentiation. After the atrophy simulation by DOX, cells were washed in PBS (BioShop, Poland) before and after fixation in 4% formaldehyde (Roth, Germany). Mitochondrially Encoded Cytochrome *C* Oxidase I (MTCO1) monoclonal antibody (1D6E1A8, cat no. 459600, ThermoFisher), prohibitin polyclonal antibody (cat no. PA5-27329, ThermoFisher), and voltage-dependent anion channel (VDAC, cat no. ab34726 Abcam) antibody were used in 1:200 dilution for the immunocytochemical reaction. According to our previous study, the immunocytochemical (ICC) assay was performed (Novickij et al., 2020). All samples were counterstained with hematoxylin (Roth, Germany) for 60 sec. A diaminobenzidine-H_2O_2 mixture stained the immunocytochemical reaction to visualize the peroxidase label. The intensity of ICC staining was evaluated as: (-) negative, (+) weak, (++) moderate and (+++) strong.

2.11. Statistical analysis

All data were expressed as the mean ± SD. Statistical significance was determined using one-way ANOVA, where values $p \leq 0.05$ or $p \leq 0.005$ were classified as statistically significant. The obtained values were related to the untreated appropriate control in the viability assay, i.e., differentiated or undifferentiated. Prism software (GraphPad Software v. 7.0) was used for the evaluation.

3. Results

3.1. Atrophy detection

In the study, C2C12 cells were differentiated to myotubes (Fig. SM1 – CTRL) and then exposed to 2 and 4 μM doxorubicin to stimulate atrophic conditions. The observations of myotubes morphology revealed that 24 h exposure to 4 μM of DOX altered cells' morphology to "not differentiated". Moreover, incubation with DOX slightly reduced cell number, which was shown in Fig. SM1- (DOX (4 μM)). Atrophic changes were examined by FoxO3 immunostaining and microscopical observations of morphology. The immunostaining results are shown in Table 1 and Fig.SM-2 in *Supplementary Material*. The validation of FoxO3 – an atrophy marker, was also performed in undifferentiated cells as a control. The obtained results revealed an intensive stained reaction after the exposure to 2 μM of DOX in 90% of cells and 100% reactive cells after the exposure to 4 μM of DOX.

3.2. Viability studies

The results obtained from the viability studies after 24 h exposure time to natural drugs and doxorubicin are shown in Fig. 2. There were used two variants of cells: undifferentiated and differentiated. The obtained results demonstrated a significant cytotoxic effect of celastrol on the cell viability with the increasing concentration (greater than 0.1 μM). In the case of the exposure to Rhynchophylline similar effect was observed. However, undifferentiated cells were more sensitive to this compound. Noncytotoxic concentrations of Ryn were in the range of 2.5–10 μM. The incubation with doxorubicin (Fig. 2c) showed that undifferentiated cells were more sensitive to DOX. The increasing concentration provoked a slight decrease in cellular viability to a minimum of c.a. 47% in undifferentiated cells and almost 70% in differentiated cells. The obtained results enabled the calculation of IC50 for all used compounds, and results are presented in Fig. 2d, and the values are shown in Table 2. In all cases, we can see that differentiated cells are less sensitive to natural compounds.

Table 1
The semi-quantitative determination of the expression of the atrophy marker - FoxO3 in C2C12 cells differentiated and undifferentiated after exposure to doxorubicin (DOX) – 24 h observations.

	undifferentiated	differentiated
Control cells	50% ± 5 +	70% ± 5 ++
2 μM DOX	5% ± 1 ++	90% ± 3* +++
4 μM DOX	10% ± 2*++	100% ± 7* ++/+++

* $p \leq 0.05$.

3.3. Atrophy neutralization in vitro

In the next stage, cells healing from the atrophic conditions was performed after the simulated atrophy induction. Firstly, differentiated cells were exposed to 4 μM DOX to induce atrophic changes. Then cells were washed by medium and exposed to celastrol (CLS) or Rhynchophylline (RYN). The results are demonstrated in Fig. 3. Undifferentiated cells were used as a negative control (Fig. 3 a and c). There was demonstrated that 0.01, and in particular 0.5 μM concentrations of CLS (Fig. 3b) protected differentiated cells, and cell viability maintained in the control cells. Higher concentrations caused a cytotoxic effect and significantly reduced cell viability (Fig. 3a). A similar effect was observed in the case of Rhynchophylline. The incubation with 2.5 and 5 μM RYN protected differentiated cells after DOX exposure, maintaining viability on the control level (Fig. 3d). Undifferentiated cells were not sensitive, and Rhynchophylline only slightly changed cell viability (Fig. 3c).

The results obtained from the analysis of myoblasts diameter are shown in Fig. 4., Fig. SM1 was used as a model for the measurements of the width and length of myoblasts. Measurements were performed with ImageJ 1.53 k software (ROI Manager, Multi Measure) (Schindelin et al., 2012). The measurement of length and width of myoblasts were represented as bars (Fig. 4 a and b). DOX exposure caused no significant changes in cells' width but a significant decrease in cells' length. Then the exposure to CLS or RHY significantly stimulated cells to lengthen. Cell lengthening was stimulated 3-fold by CLS, and 2-fold by RHY.

Further, MAFbx labeled antibody was used as an atrophy marker, where a specific translocation between cytoplasm and nuclei can be noted (Lagirand-Cantaloube et al., 2012). The atrophy marker - MAFbx expression results in differentiated C2C12 cells after the exposure to doxorubicin (DOX) are shown in Fig. 5 and Table 3. A reduced expression of MAFbx in cells exposed to Rhynchophylline (on control level) and strong nuclear expression in cells after treatment with celastrol were observed. Fig. 5b demonstrates the mean fluorescence intensity values calculated in ImageJ software. The highest signal corresponding to MAFbx expression was detected for cells exposed to DOX and celastrol, and the signal appeared mainly in nuclei. Cells exposed to 4 μM of DOX revealed only some increase in the fluorescent signal. Rhynchophylline caused a more substantial decrease in the fluorescent intensity in cells.

3.4. Mitochondrial markers in cells counterbalanced from the simulated atrophic state.

According to the viability experiments, we have selected noncytotoxic concentrations: 10 nM for celastrol and 5 μM for Rhynchophylline for mitochondrial markers evaluation. Mitochondrial markers determined the energetic state of treated cells, which is significant in cell regeneration, particularly in degenerated (atrophic) muscle cells. Mitochondria play a crucial role in the meta-

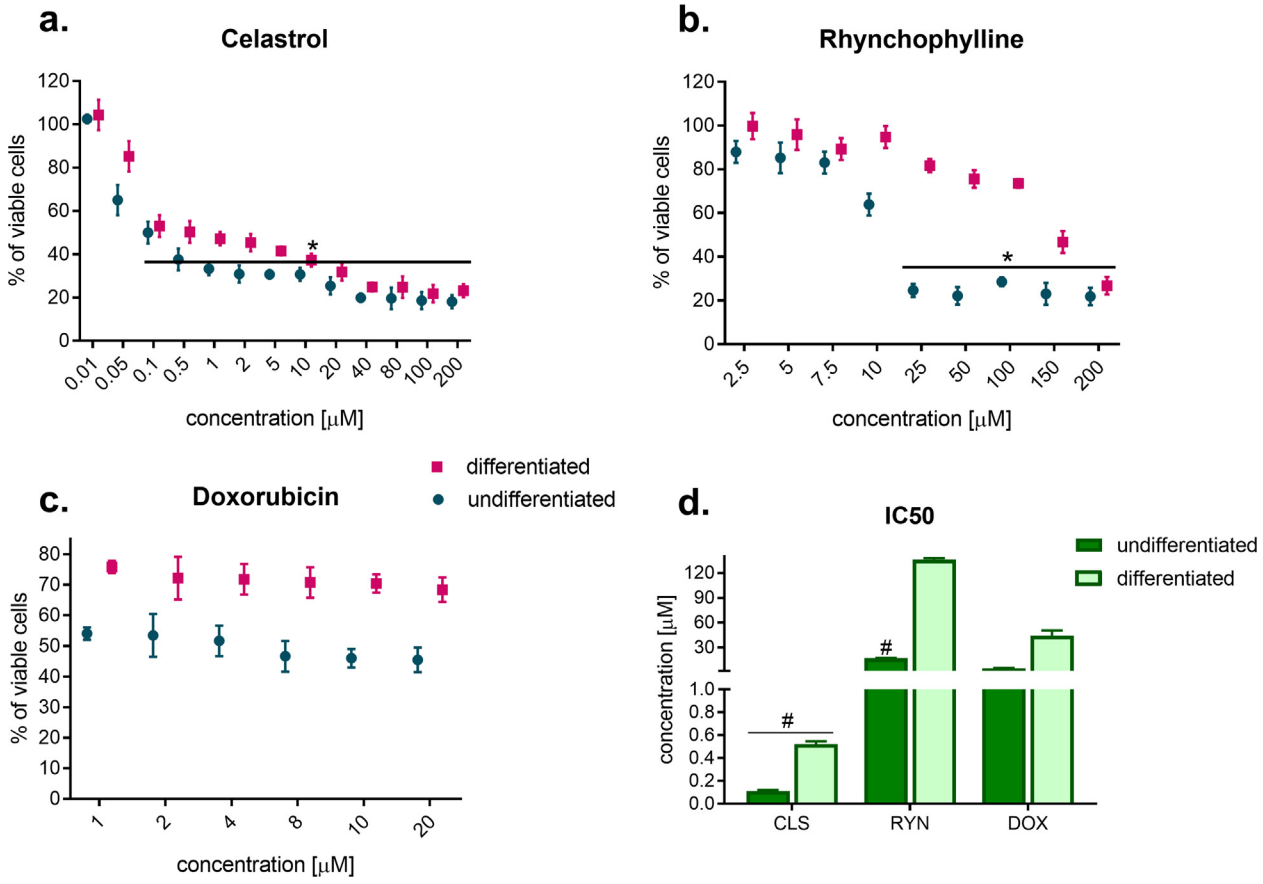

Fig. 2. The evaluation of the cells' proliferation in undifferentiated and differentiated C2C12 cells after 24 h exposure to (**a**) celastrol, (**b**) Rhynchophylline, and (**c**) doxorubicin, (**d**) graphical representation of IC50 Values marked (* or #) were classified as statistically significant $p \leq 0.05$.

Table 2
IC50 values [μM] were calculated based on the viability results.

C2C12 cells	Celastrol	Rhynchophylline	Doxorubicin
undifferentiated	0.110* ± 0.012	17.068* ± 0.419	5.148 ± 0.217
differentiated	0.521* ± 0.025	135.810 ± 2.134	44.469 ± 6.037

* $p \leq 0.05$.

bolism of muscle cells, energy resources, and regulation of the signaling and production of reactive oxygen species, calcium homeostasis, and cell death regulation (Gouspillou and Hepple, 2016). The cell healing process from stressed conditions should reveal an increasing energetical condition of mitochondria and their markers. Thus VDAC1 (Voltage-Dependent Anion Channel), prohibitin (protein located in the inner membrane of mitochondria), and MT-CO1 (cytochrome *c* oxidase) were immunohistochemically determined as markers. The results are shown in Fig. 6 and Table 4. There was observed that doxorubicin and exposure to natural drugs induced an increased immunostained reaction of MT-CO1, VDAC, and prohibitin. The exposure to 4 μM doxorubicin induced a partial immunostained reaction of MT-CO1 and prohibitin in cells and a stronger reaction in the case of VDAC expression in 100% of cells. The treatment of cells with celastrol intensified the immunoreaction with MT-CO1 in the cytoplasmic area. VDAC immunoreaction was less intensive but observed in 90% of cells. Prohibitin expression revealed the most intensive stained reaction in ca. 100% of cells. The exposure to Rhynchophylline demonstrated weaker immunoreaction of MT-CO1 in 90% of cells, an intensive stained reaction in the case of VDAC (98%), and a slightly weaker expression of prohibitin (++).

4. Discussion

The available studies indicate that skeletal muscle atrophy involves complex molecular signaling that is still unclear (Ebert et al., 2019). Mitochondrial dysfunction had been directly linked to muscle wasting; thus, the idea of mitochondrial dysfunction contributes also to disuse muscle atrophy (Hyatt et al., 2019). The most promising therapies are based on troponin activation or gene therapy, where small molecules such as RG7916, LMI070 are applied (Hyatt et al., 2019)(Jędrzejowska and Kostera-Pruszczyk, 2020)(Prior et al., 1993). However, this approach is still limited to a broader range of patients. Another problem may be the various sensitivity of atrophic cells to the treatment, which are at different stages of differentiation. Our observations revealed that undifferentiated cells were more sensitive to both natural compounds. It can be related to the difference between the differentiated and undifferentiated types of cells. In turn, some authors

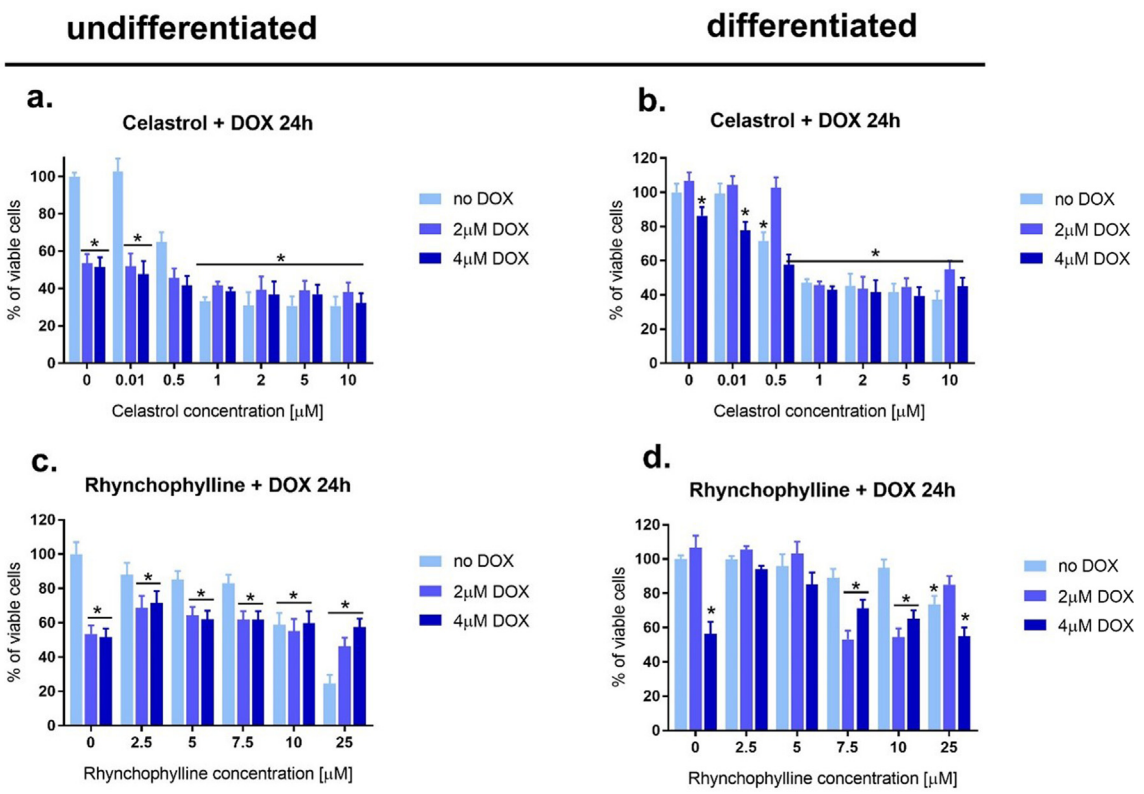

Fig. 3. The evaluation of the cells' proliferation in undifferentiated (**a**, **c**) and differentiated (**b**, **d**) C2C12 cells after exposure to doxorubicin (24 h) and counterbalancing by celastrol and Rhynchophylline. "no DOX" corresponds to cells not exposed do doxorubicin. Values marked (*) were classified as statistically significant $p \leq 0.05$.

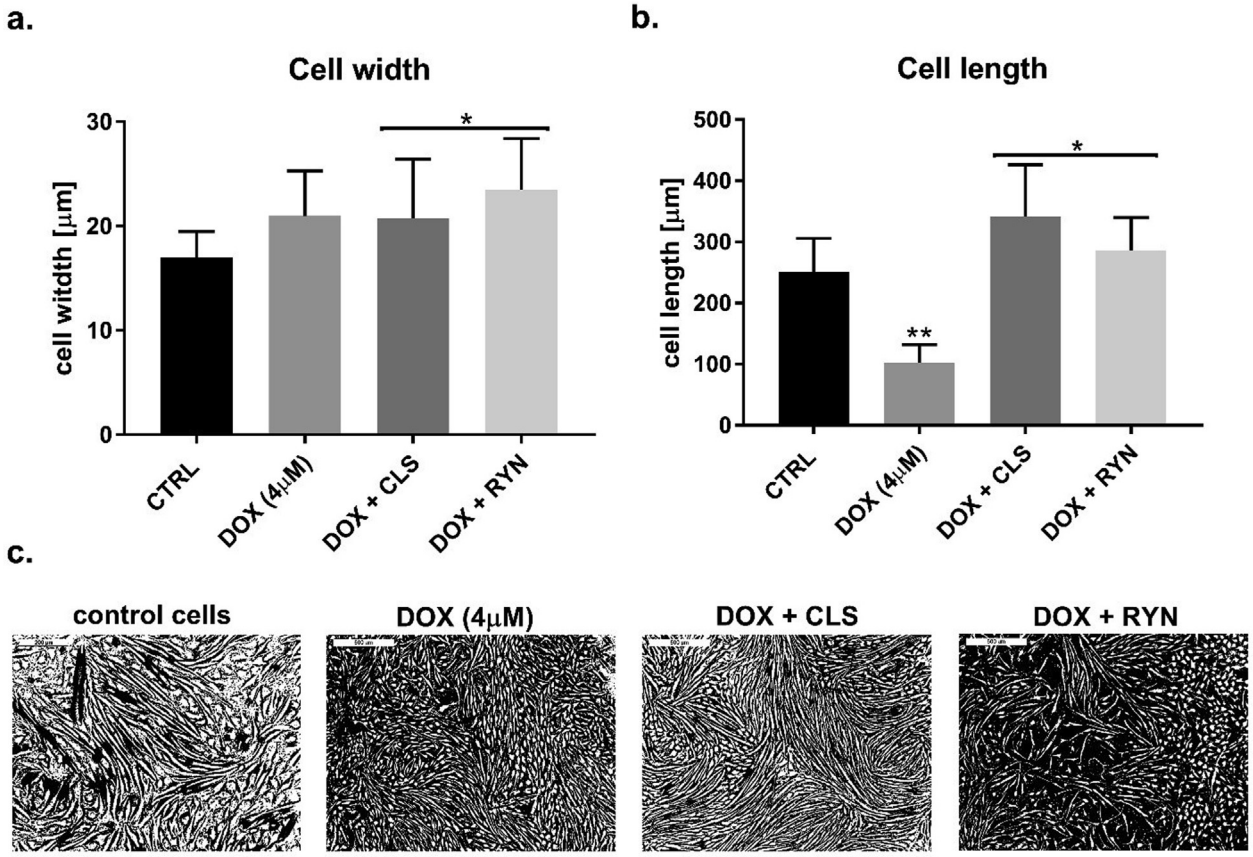

Fig. 4. The evaluation of the muscle cell diameter (**a**) width and (**b**) length in [μm]. Black and white transformations (**c**) were used for the image analysis. Control cells correspond to differentiated C2C12 cells after 5 days (scale bar 200 μm); DOX (μM) corresponds to cells exposed to doxorubicin (24 h) after differentiation (scale bar 500 μm); DOX + CLS – cells counterbalanced by celastrol (10 nM) or DOX + RYN by Rhynchophylline (5 μM) (scale bars 500 μm). Values marked (*) were classified as statistically significant $p \leq 0.05$ or (**) $p \leq 0.005$.

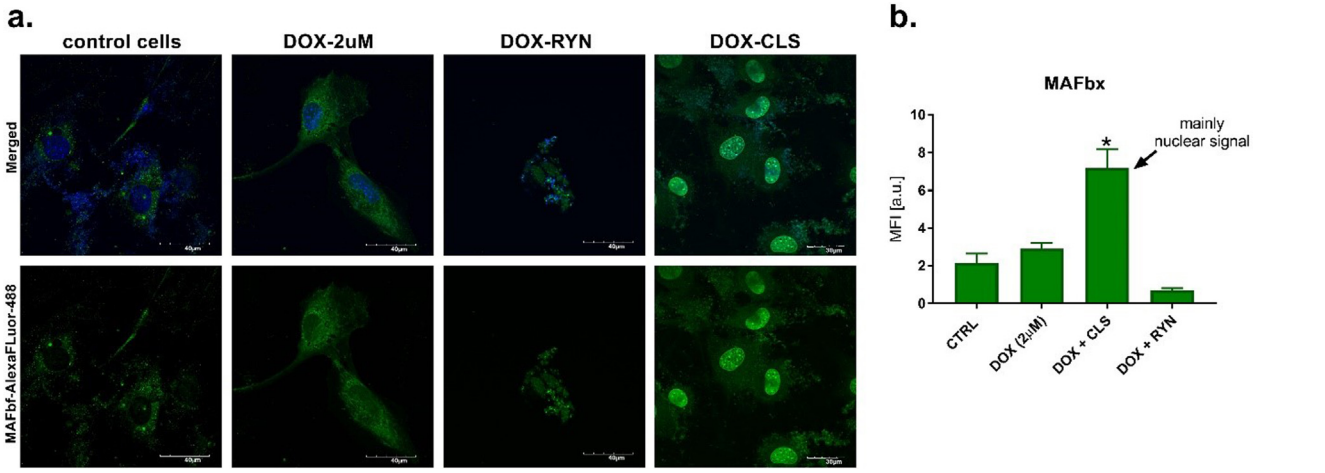

Fig. 5. The immunofluorescent evaluation of the atrophy marker – (**a**) MAFbx in differentiated C2C12 cells after 24 h exposure to doxorubicin (DOX – 4 μM), and exposed to celastrol (10 nM), or Rhynchophylline (5 μM). (**b**) The mean fluorescence intensity values evaluated based on the immunofluorescent studies of the atrophy marker - MAFbx expression in differentiated C2C12 cells after exposure to doxorubicin (DOX- 4 μM). Values marked (*) were classified as statistically significant $p \leq 0.05$.

Table 3
The semi-quantitative determination of the atrophy marker - MAFbx expression in differentiated C2C12 cells after doxorubicin (DOX- 4 μM) exposure.

	Differentiated C2C12 cells
Control cells	70% ± 5 +/++
2 μM DOX	96% ± 4 ++ (reduced cell number and structure)
DOX + celastrol (10 nM)	92% ± 8* +++Nuclear localization
DOX + Rhynchophylline (5 μM)	90% ± 10 +/++

* $p \leq 0.05$.

observed that differentiated C2C12 cells in adherent culture are more resistant to apoptosis, which is related to the increased expression of Bak and Bad in this type of culture (Schöneich et al., 2014). Additionally there was proved that differentiated cells have higher expression of potassium channels TASK2 and TREK1, which play a significant role in the maintenance of basic cellular parameters (Afzali et al., 2016). The available data also indicate that both types of cells have various protein metabolism, which also determines drug response (Cardin et al., 2017). The important role in the differentiation process play the upregulation of genes such as sarcoglycan and myoglobin that encode essential functional components of skeletal myotubes (Szustakowski et al., 2006). Tannu et al. demonstrated that 75 proteins are regulated during the phenotypic transformation of C2C12 myoblasts into fully differentiated, multi-nucleated, and post-mitotic myotubes. Additionally, the authors discovered a differential accumulation of 26 phospho-proteins in C2C12 differentiating cells (Tannu et al., 2004). Thus, this changing profile of genes and proteins undoubtedly determines the sensitivity or the resistance to the applied treatment.

In this current study, we proposed the application of the natural compounds: celastrol and Rhynchophylline for counterbalancing from atrophic conditions, cells triggered by doxorubicin. There was noted that validated doxorubicin concentrations can cause a subacute decrease in muscle cell volume in mice and humans and provoke atrophy dependent on MuRF1 (Muscle RING-finger protein-1) (Willis et al., 2019). We have used marked atrophy with FoxO3 before therapy and with MAFbx after treatment, which are crucial in determining the appropriate level of simulated atrophy and, finally, the efficacy of regenerative activity of natural compounds. Or results show that Rhynchophylline decreased MAFbx immunofluorescent signal and simultaneously stimulated differentiated cells for increased proliferation. However, in the case of celastrol treatment, nuclear distribution of MAFbx was observed in differentiated cells. Despite the relatively high increase in cell viability (ca. 130%), it might signal atrophic conditions. There is known that the MAFbx overexpression in myotubes generates atrophy (Ebert et al., 2019),(Zhang et al., 2013). Some authors also found that MAFbx contains two functional nuclear localization signals (NLS) (Lagirand-Cantaloube et al., 2012), which might also be related to the resistance to the atrophic changes. Up to now, only an increased cytoplasmatic MAFbx distribution was related to the atrophic state of the cells. The role of the nuclear MAFbx distribution is still not clarified and requires further investigations. Atrophy can also be validated by the myoblasts measurements (Oelkrug et al., 2015)(Bass et al., 2021). Here myoblasts diameter was also measured to assess neutralization effects stimulated by natural drugs. There was observed that simulated atrophic cells recovered to myotubes again when treated with celastrol or Rhynchophylline. In relation to the other studies, the results obtained in this study can be associated with the healing process from atrophic conditions. Gwag et al. showed that celastrol had cytoprotective properties and inhibited Akt1 and ERK1/2 pathways in C2C12 cells (Gwag et al., 2013). Another study revealed that celastrol-induced HSP72 overexpression overcomes the atrophic silencing effect of the HSP72 gene by increasing FoxO3 phosphorylation and activating the Akt1-ERK1/2 signaling pathway (Gwag et al., 2013). In the other studies, the reducing effect of celastrol was proved in diseases associated with neurodegenerative dysfunctions. Authors indicated the usability of this compound in the therapy of muscular atrophies (Bai et al., 2021). The anti-arthritic effects of celastrol in rheumatoid arthritis (RA) were demonstrated by Kam Wai Wong et al. There was proved that celastrol triggered Ca^{2+} signaling (Wong et al., 2019), and also efficiently induced paraptosis, apoptosis, and autophagy in cancer cells (Wang et al., 2012). The next cause of muscle atrophy is mitochondrial dysfunction (Hyatt et al., 2019). Mitochondria are crucial organelles, particularly in excitable cells with high energy demand (Glancy et al., 2015). Our results indicated that celastrol stimulated mitochondrially encoded cytochrome *C* oxidase I, and prohibitin. Doxorubicin also provoked immunostained reactions in differentiated cells, which can be related to a response to oxidative stress. The main difference was associated with the intracellular distribution and intensity of the reaction of detected mitochondrial markers. For example, in cardiac cells (H9C2), doxorubicin had no significant

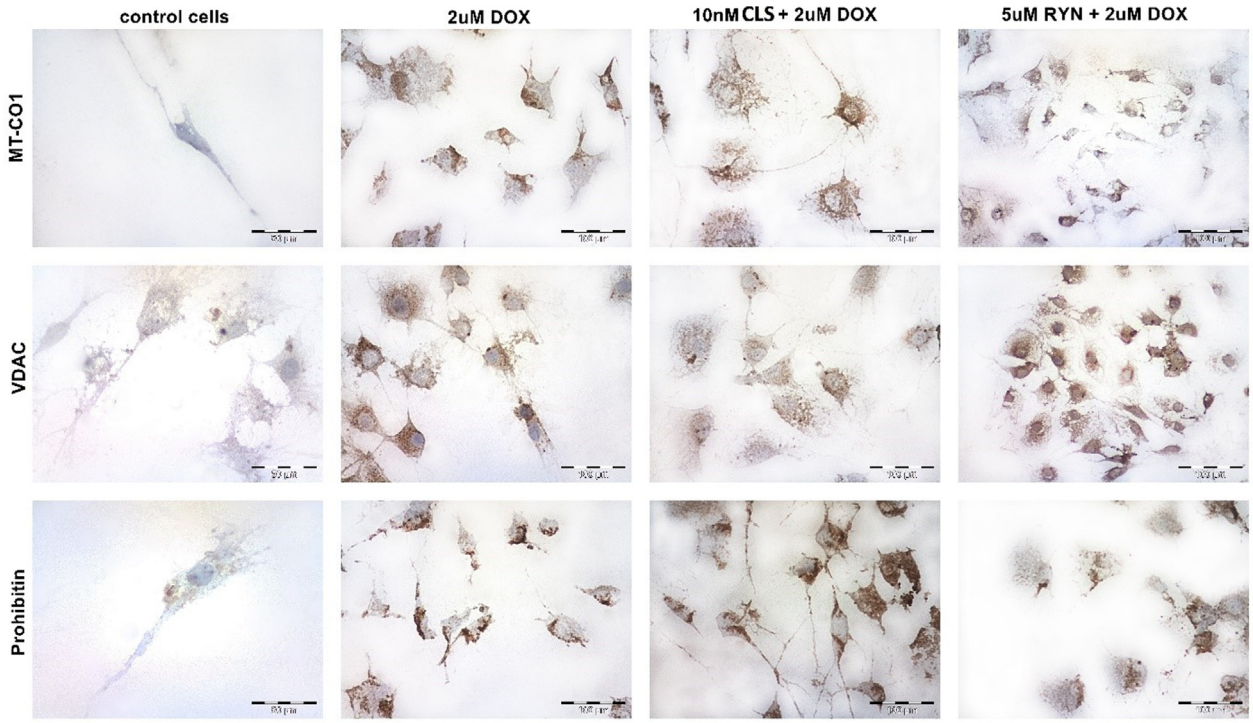

Fig. 6. The immunocytochemical staining of MT-CO1, VDAC, and prohibitin in differentiated C2C12 cells after exposure to doxorubicin (DOX) and treatment with celastrol (10 nM, CLS) and Rhynchophylline (5 μM, RYN).

Table 4
The semi-quantitative determination of the immunocytochemistry results of MT-CO1, VDAC, and prohibitin in differentiated C2C12 cells after exposure to doxorubicin (DOX) and treatment with celastrol and Rhynchophylline.

	MT-CO1	VDAC	Prohibitin
Control cells	10% ± 2 +	25% ± 4 ++	15% ± 3 +/++
DOX	90% ± 5 ++	90% ± 10 ++	95% ± 5 ++
DOX + celastrol (10 nM)	95% ± 5 +++	90% ± 5 ++	96% ± 4 ++
DOX + Rhynchophylline (5 μM)	90% ± 5 ++	98% ± 2 +++	97% ± 3 ++

effect on prohibitin levels (Qureshi et al., 2015). In turn, Rhynchophylline promoted the expression of VDAC and prohibitin more intensively. Rhynchophylline was previously noted as a modulator of mitochondrial mechanisms. In myocardial ischemia–reperfusion cardiomyocytes, Ryn also modified Ca^{2+} and MMP (mitochondrial membrane potential) levels. Additionally, Rhynchophylline decreased the oxidative stress and degree of mitochondrial permeability transition pore (mPTP) in excitable cells (Qin et al., 2019). The available data indicated that Rhynchophylline also affects the sleeping process through ion channel mechanisms (Ballester Roig et al., 2021).

5. Conclusions

Atrophy-related diseases have different backgrounds, including genetic (e.g., SEM), or mechanical injury of the spinal cord. The use of natural compounds can bring a possible economic effect and, what is more, a supportive method for inhibiting disease progression. Here we have used an *in vitro* model of atrophic muscles, which enabled us to verify a possible alternative or supportive method using non-usual natural substances such as celastrol and newly presented Rhynchophylline. The obtained results demonstrated that natural drugs validated in this study can be good protectants and weaken the effects in the atrophic tissues. It should be considered an application of natural compounds as significant support in atrophy-related diseases. Additionally, the created atrophy simulation model *in vitro* and proposed natural drugs for regeneration can be helpful in further pharmacological studies on more advanced human-related models.

CRediT authorship contribution statement

Maksymilian Wastag: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization. **Katarzyna Bieżuńska-Kusiak:** Methodology, Investigation, Data curation, Visualization. **Anna Szewczyk:** Methodology, Investigation, Visualization. **Wojciech Szlasa:** Methodology. **Bożena Grimling:** Writing – review & editing. **Julita Kulbacka:** Conceptualization, Validation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2022.06.008.

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