

# Effect of *Uncaria tomentosa* aqueous extract on the response to palmitate- induced lipotoxicity in cultured skeletal muscle cells

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## Research Article

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# Abstract

**Introduction:** Type 2 diabetes mellitus (T2DM) is frequently associated with dyslipidemia, which corresponds to the increase in the triglycerides and fatty acid concentrations in tissues, such as the skeletal muscle. The use of herbal medicines as *Uncaria tomentosa* (Ut) has been proposed as an auxiliary treatment for patients with T2DM. In this study, it was evaluated the Ut aqueous extract effect on cell viability of skeletal myoblasts from C2C12 lineage exposed to the free fatty acid palmitate (PA), and on the reactive oxygen species (ROS) production, which consists a central event involved in T2DM pathogenesis.

**Methods:** Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), at 37°C humidified atmosphere and 5% CO<sub>2</sub>. The cells were incubated with PA in different concentrations, in the presence or absence of 250 µg/ml Ut aqueous extract, for 2, 6 or 24 h. After these periods, oxidative stress was evaluated by fluorescence spectroscopy.

**Results:** Incubation of cells with PA and Ut aqueous extract resulted in an increase of, at least, 50% in cell viability compared to control with only PA. The treatment of cells with Ut aqueous extract, for 6 h, followed by exposure to 500 µM PA, caused 38% less ROS formation than those incubated with only the free fatty acid.

**Conclusion:** The Ut aqueous extract promoted a rise in cell viability, reduced cell death and attenuated ROS formation in cultures incubated with 500 µM PA, protecting cells from the fatty acid lipotoxicity.

## 1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic non-communicable disease with increasing prevalence in recent years (Bourne et al, 2013; WHO, 2020), resulting in low quality of life. It is estimated that in 2045 there will be an increase of 35% in cases worldwide, exceeding 690 million (Chao et al., 2019; IDF, 2017; Zhao et al., 2015).

T2DM is considered a multifactorial syndrome characterized by plasma chronic hyperglycemia with disorders in the metabolism of carbohydrates, lipids and proteins, resulting from changes in insulin production, secretion and/or its mechanism of action of insulin. In addition, T2DM is often associated with dyslipidemia (WHO, 2009; Giuffrida et al., 2017; Cui et al., 2020).

Basically, diabetic dyslipidemia consists of increasing concentrations of triglycerides, which are composed of a glycerol molecule associated with three fatty acid molecules (Muniz et al., 2012; Fung & Berger, 2016). Following the lipolysis process, the free fatty acids (FFA) are transported in the blood, mostly, reversibly linked to albumin and it is, mainly used by the skeletal and cardiac muscle tissue as an energy source (Lemaire et al., 2000; Almeida et al., 2014).

In the skeletal muscle, the accumulation of fatty acids such as palmitate (PA), which represents about 70 to 80% of the total plasma FFA (Hoehn et al., 2008; Lambertucci et al., 2008), stimulates the production of pro-inflammatory cytokines and reactive oxygen species (ROS) (Ly et al., 2017; Lefranc *et al.*, 2018). In addition, the increased concentration of palmitate promotes mitochondrial dysfunction, which also leads to ROS production and oxidative stress (Ly et al., 2017; Guo et al., 2020).

It has been shown the involvement of ROS and cellular redox mechanisms in the regulation of molecular processes (Ly et al., 2017; Franchina et al., 2018; Guo et al., 2020). In general, the antioxidant enzymes present in the tissues are sufficient to block the damage caused by physiological amounts of ROS. However, an imbalance between the formation of ROS and the ability of cells to remove these compounds results in the oxidative stress (Oter et al., 2012; Zhang et al., 2019). Particularly, oxidative stress has been related to insulin resistance (IR) observed in T2DM (Ly et al., 2017), a condition in which cells do not respond adequately to circulating insulin levels. Although many molecular events can be related to IR, mostly are direct or indirectly associated with oxidative stress and inflammation.

Considering the molecular events involved in oxidative stress, the use of antioxidants combined with drugs that target lipid metabolism has been suggested for the treatment of obese individuals with T2DM.

Currently, it has been a challenge for health professionals and consumers to make decisions regarding the safe combinations of drugs and medicinal plants, due to a lack of strong clinical evidence related to plant compounds interactions. In addition, little is known about the mechanisms involved in these processes (Zhang, 2017; Feltrin and Simões, 2019).

In 2013, WHO (World Health Organization) published the “WHO traditional medicine strategy: 2014–2023”, which aims to support the practice of complementary and alternative medicine to promote public health, including phytotherapy, which is the use of medicinal plants for a healthy application. The plan aims to promote safety, efficacy, and quality of complementary and alternative medicine by expanding the knowledge base and providing guidance under the regulatory and quality assurance standards (WHO, 2013). In 2016, the American National Center for Complementary and Integrative Health (NCCIH) published a strategic plan to explore the science of complementary and integrative health, including phytotherapy (NIH, 2016). In this context, several plants of the Brazilian flora have been explored focusing on therapies for the treatment of different diseases.

*Uncaria tomentosa* (Ut), popularly known as “cat's claw”, is a plant species of the Rubiaceae family recognized as an herbal medicine that appears in the National List of Essential Medicines (RENAME) (Brazil, 2018) for being used in capsules, gels or teas.

It has been already shown in *in vitro* tests that the Ut bark aqueous extract presents antioxidant activity (Azevedo et al., 2017; Navarro-Hoyos et al., 2018). Other studies have also shown the presence of antioxidant compounds such as cinchonain, a molecule belonging to the proanthocyanidin class (Wirth & Wagner, 1997; Akesson *et al.*, 2003; Heitzman et al., 2005). In this sense, using a cell culture model, Bors et al. (2012) confirmed the antioxidant effect of Ut leaves aqueous preparations on human erythrocytes

against oxidative stress. It has also been shown that the Ut aqueous extract prevents the lipid peroxidation in phagocytic cells and the consequent damage to the cell membrane and DNA (Gonçalves et al., 2005; Bors et al., 2012).

Based on these evidences and the fact that skeletal muscle tissue is an important target for the high plasma triglycerides and FFA content, which are related to diabetic dyslipidemia and lipotoxicity, we developed an *in vitro* model that mimics the lipotoxic scenario associated with T2DM. We have shown that the viability of muscle cells incubated with 500  $\mu$ M PA, for 24 or 48 h, has been reduced to approximately 44 and 26% in 24 and 48 h, respectively (Freitas et al., 2020). On the other hand, the treatment with 250  $\mu$ g/ml Ut aqueous extract resulted in an increase of 50% on cell viability compared to control (Freitas et al., 2020). Considering the convergence of multiple processes such as cell death, oxidative stress, dyslipidemia and T2DM, we studied the effect of Ut aqueous extract on the palmitate-induced oxidative stress in skeletal muscle cells under physiological and lipotoxic concentrations.

## 2. MATERIAL AND METHODS

### 2.1 Cell cultures

The skeletal muscle cell line C2C12 (ATCC - American Type Culture Collection, CRL – 1772) was acquired from Rio de Janeiro Cell Bank (BCRJ). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM glucose, 10% fetal bovine serum (FBS) and the antibiotics 10,000 IU/ml penicillin and 10 mg/ml /streptomycin, in 5% CO<sub>2</sub> incubator at 37°C, until the culture reach 80% of confluence. At that time, cells were transferred to 6 or 96 well plates for the different experiments.

### 2.2 Ut aqueous extract and palmitate solutions preparation

The powder of *Uncaria tomentosa* crude extract (Reference number 100001372, code 2470), from the plant root bark, was donated by Herbarium Botanic Laboratory (herbarium.com.br, Curitiba, PR, Brazil). A powdered fraction was used to prepare the aqueous extract. The solution was prepared weekly, using 100 mg of the extract in 1 ml of distilled water (dH<sub>2</sub>O), which was kept at 37°C for 24 h according to Bors et al. (2012). After this period, the solution was homogenized and centrifuged for 5 min, at 400 x g. The supernatant was recovered and filtered (0,22  $\mu$ m) to be used in the experiments. The chromatogram of the crude extract, sent by the supplier (Supplementary Information S1), confirms the presence of uncarine D, uncarine F, mitraphylline, isomitraphylline, uncarine C and uncarine E compounds.

The PA stock solution was prepared at 0,1 M concentration in absolute ethanol. At the moment of incubating with cells, stock solution was diluted and 2% bovine serum albumin (BSA) was added to conjugate the fatty acid to the protein.

### 2.3 Cell treatments

Cells were plated at 2x10<sup>5</sup> or 1x10<sup>4</sup> cells/ml density, in 6 or 96-well plates, respectively, and incubated with PA solution in different concentrations (0, 10, 50, 100, 250, 500, 750 and 1000  $\mu$ M), for 24 or 48 h, in

the absence or presence of Ut aqueous extract pre-incubation, in different concentrations (0, 10, 50, 100, 250, 500, 750 and 1000 µg/ml), for 2, 6 or 24 h, as indicated in each experiment.

To verify whether the effect of Ut aqueous extract against PA cytotoxicity was related to its presence in the intracellular or extracellular environment, the following experimental approaches were adopted, as shown in Fig. 1: A) after the pretreatment, for 2 or 6 h, with Ut aqueous extract, cultures were washed with PBS before incubation with PA in different concentrations, for 24 h (preventive condition); B) after the pretreatment, for 2 or 6 h, with Ut aqueous extract, cultures were incubated with PA in different concentrations, for 24 h; C) after the incubation with PA in different concentrations for 24 h, cultures were washed with PBS and then the Ut aqueous extract was added, for 2 or 6 h (therapeutic condition).

## 2.4 Cell viability determination

The 3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock solution was prepared at 5 mg/ml in PBS. After the incubation with PA for 24 h, in the absence or presence of Ut aqueous extract pretreatment, 5 µl of MTT solution were added to the cultures in 96-well microplate and the cells were cultured for 4 h, at 37°C. After this period, the medium was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. Cell viability was determined by the absorbance at 595 nm in the BioTek Synergy HT microplate reader. Four individual experiments were performed in triplicates and the results were analyzed with Gen5 software. The absorbance was converted into the percentage (%) of viable cells in relation to the total, as following:

$$\text{Total \%} = \left( \left[ \frac{\text{Abs (sample)}}{\text{Abs (control)}} \right] \times 100 \right) - 100$$

The degree of cytotoxicity was determined using the classification by Oliveira (2009).

## 2.5 Cell viability and DNA fragmentation analysis by flow cytometry

After the incubation with PA for 24 h, in the absence or presence of Ut aqueous extract pretreatment, cells were collected with 0.25% trypsin. The cell suspension was centrifuged for 5 min, at 400 x g. To assess cell viability, the pellet was resuspended in 200 µl of PBS and 5 µl of 1 mg/ml propidium iodide (PI) was added to the cell suspension. For DNA fragmentation analysis, the pellet was resuspended in 300 µl of PBS containing 0.1% Triton® X-100 and 20 µg/ml PI. Cells were evaluated on Guava EasyCyte 8HT flow cytometer using the InCyte® software. Three individual experiments were performed in triplicates and 10.000 events were acquired per sample. The results were expressed as a percentage of the total number of cells in each sample.

## 2.6 Intracellular ROS production determination

After cell incubation with 100 or 500  $\mu\text{M}$  PA, in the absence or presence of Ut aqueous extract pretreatment in 96-well microplates, the ROS production was quantified by the fluorescent marker 2',7'-dichlorofluorescein diacetate (DCFDA) using the commercial kit DCFDA/HeDCFDA d6883 (Sigma-Aldrich, St. Louis, USA). The medium was removed from the plates and two washings with PBS were performed. Then, 10  $\mu\text{M}$  DCFDA diluted in DMEM without phenol red was added and the incubation continued for 30 min at 37°C. Cultures were washed twice with PBS and the ROS presence was evaluated at  $\lambda_{\text{excitation}} = 485 \text{ nm}$  and  $\lambda_{\text{emission}} = 528 \text{ nm}$  in the BioTek Synergy HT microplate reader. Three individual experiments were performed in triplicates and the results were analyzed using the Gen5 software (version 1.0.14, BioTek). The fluorescence values were expressed in Fluorescence Arbitrary Units (FAU) and converted into percentage, considering the number of viable cells in each sample, in relation to the control (as shown in 2.4 topic).

## 2.7 Statistical analysis

The results were expressed as the mean  $\pm$  standard error (SE) and the differences were analyzed by two-way ANOVA, followed by the Bonferroni post-test, using the GraphPad Prism software (version 8). The differences were considered significant at  $p < 0.05$ .

## 3. RESULTS

### 3.1 Determination of cell viability

Cell viability was evaluated in cultures exposed to PA in different concentrations. It was observed that the culture incubation with 250  $\mu\text{M}$  PA resulted in the reduction of viability to approximately 73% in 24 h and 50% in 48 h. In addition, cell viability was reduced to approximately 44 and 26%, in the incubation by 24 or 48, respectively, with PA in concentrations from 500  $\mu\text{M}$  when compared to the control (cells cultured in the presence of 0,05% ethanol) (Fig. 2). However, this FFA was not toxic at the concentrations of 10, 50 and 100  $\mu\text{M}$ .

Also, it was possible to verify that the incubation with PA in concentrations higher than 250  $\mu\text{M}$ , for 48 h, resulted in reduction of cell viability. Specifically, incubation with 250  $\mu\text{M}$  PA for 48 h caused a decrease in cell viability of 32% compared to the incubation for 24 h, showing a time-dependent effect. At concentrations higher than 250  $\mu\text{M}$ , cell viability was reduced by approximately 59%, when compared to the incubation period of 24 h.

The incubation of cells with only Ut aqueous extract, resulted in significant increase in cell viability between 30 and 70% at concentrations from 250  $\mu\text{g/ml}$  compared to the control (Fig. 3). Since the concentration of 250  $\mu\text{g/ml}$  was the lowest capable of promoting an increase in cell viability in comparison to control, this was the one chosen for further experiments.

As observed in Fig. 2, it was shown that PA is cytotoxic from the concentration of 250  $\mu\text{M}$  when incubated for 24 or 48 h with the cells, so this concentration was used in the following investigations.

Interestingly, the pretreatment with 250 µg/ml Ut aqueous extract, for 2 or 6 h, and subsequently, with 250 µM PA, resulted in increase of cell viability of 21 and 30%, respectively, when compared to cultures incubated with only the FFA (Fig. 4A). This same effect was observed in cultures incubated with 500, 750 and 1000 µM PA, in which the pretreatment with 250 µg/ml Ut aqueous extract, for 2 or 6 h, promoted an increase in cell viability between 50 and 80%, respectively, compared to cultures incubated with only PA.

The cell viability in cultures pretreated with the 250 µg/ml Ut extract, for 2 or 6 h, washed with PBS to remove the Ut extract from the medium and, subsequently, incubated with 250 µM PA presented an increase of by 15 and 20% in cell viability, respectively, compared to the cultures incubated with only this FFA at the same concentration (Fig. 4B). In cultures incubated with PA in concentrations above 500 µM, the pretreatment with the Ut aqueous extract, for 2 or 6 h, resulted in increase in cell viability between 30 and 70%, respectively, in comparison to control samples.

Regarding to the period of time of pretreatment with the Ut extract, it was shown that the incubation for 6 h resulted in a significant cell viability increase in relation to the treatment for 2 h, both when the extract was incubated concomitantly with the FFA (Fig. 4A), as well as when it was removed before the addition of PA (Fig. 4B). Indeed, it was shown that the action of Ut extract, in cultures incubated with PA at concentrations higher than 500 µM, for 6 h, increased cell viability in 11% compared to 2 h pretreatment period. Also, Ut extract promoted a raise of 21% in cell viability when incubated for 6 h, compared to incubation period of 2 h for the same PA concentrations.

A)

## 3.2 Evaluation of cell viability and DNA fragmentation by flow cytometry

The treatment of cells with only 250 µg/ml Ut extract for 2, 6 or 24 h did not cause cell death in all periods of time, compared to the control (Fig. 5). However, when the cultures were pretreated with the extract for 2 h in the preventive condition, and subsequently exposed to 500 µM PA, there was an increase of 12% in cell viability compared to the incubation with only the FFA. Also, when the cells were incubated with 500 µM PA and treated with Ut extract for 6 h, in both conditions (preventive or therapeutic), it was observed an increase of 27 and 70% in cell viability respectively, in relation to the cultures incubated with only the FFA.

In order to study molecular events related to palmitate-induced cell death, the DNA fragmentation was evaluated, as an indicative of apoptosis occurrence. In the absence of cytotoxic stimulus, the Ut aqueous extract had no effect on DNA fragmentation in cells compared to the control (Fig. 6). In cultures incubated with 500 µM PA, the percentage of cells with fragmented DNA was 60%, which is statistically different from the control. Furthermore, in cultures incubated with 500 µM PA and treated with the Ut aqueous extract, for 6 h, in the preventive or therapeutic condition, a reduction of 16% and 57% were

observed, respectively, in the number of cells with fragmented DNA, compared to cultures incubated with only PA.

### 3.3 Determination of ROS intracellular production

The incubation of cultures with 100  $\mu\text{M}$  PA, in the absence or presence of Ut aqueous extract pretreatment, did not change the percentage of ROS formation, in relation to the cultures incubated with only the FFA (Fig. 7). However, the incubation of cultures with 500  $\mu\text{M}$  PA, for 24 h, increased 20-fold the ROS formation compared to the control, indicating its involvement in ROS generation and, potentially, in oxidative stress.

Interestingly, the treatment with Ut aqueous extract, for 2 h, in the preventive or therapeutic conditions, resulted in reduction of 12 and 20%, respectively, in ROS production when compared to cultures treated with only 500  $\mu\text{M}$  PA, while the treatment with Ut extract, for 6 h, both in the preventive or therapeutic conditions, promoted a decrease of 21 and 55%, respectively, in the ROS formation, compared to control with only the FFA.

## 4. DISCUSSION

Considering the convergence of multiple processes such as cell death, oxidative stress, inflammation, dyslipidemia, IR and DM2, and that the skeletal muscle tissue is an important target for the high content of plasma triglycerides and FFA, several experimental models have been developed to evaluate the PA lipotoxicity using rat femoral and mesenteric arteries or mice isolated skeletal muscle cells (Lima and Silveira, 2018; Vorn and Yoo, 2019; Sadeghi et al., 2020).

Here, we developed a simplified *in vitro* model to evaluate the cytotoxic effect of PA on skeletal muscle cells of the C2C12 lineage. PA cytotoxicity was studied at concentrations that can be achieved during overnight fasting (100  $\mu\text{M}$ ), prolonged fasting (250  $\mu\text{M}$ ) or in uncompensated T2DM (1000  $\mu\text{M}$ ) (Chatham, 1999; Hirata & Crespo, 2002). We have shown that PA from the concentration of 250  $\mu\text{M}$  was cytotoxic to myoblasts (Freitas et al., 2020). Similarly, Filippello et al. (2018) demonstrated that the incubation of murine intestinal cells with palmitate at concentrations higher than 500  $\mu\text{M}$ , for 24 or 48 h, resulted in reduction of cell viability of 40 and 60%, respectively, in a time and concentration-dependent manner. In addition, the incubation of cultures with PA resulted in a significant rise in the number of cells with fragmented DNA, which is an indication of apoptosis occurrence (Nunes et al., 2005; Patková et al., 2014). It was also shown that the incubation of cultures with 500  $\mu\text{M}$  PA for 24 h increased 20-fold the ROS formation, compared to the control, confirming its involvement on ROS production and, potentially, in the oxidative stress.

On the other hand, the treatment with 250  $\mu\text{g/ml}$  Ut aqueous extract promoted an increase of 50% in cell viability compared to control. Comparable results were obtained by Ciani et al. (2018) using immortalized skin cells, which showed that the incubation with Ut aqueous extract at concentrations from 250 to 1000  $\mu\text{g/ml}$ , for 24 h, promoted an increase in cell viability between 40 and 60%.

We have tried different approaches to evaluate the Ut aqueous extract effect on myoblasts viability. In this sense, when the cultures were treated with Ut aqueous extract, for 2 or 6 h, followed by the incubation with PA, we found increase in cell viability by 55%, in average, when compared to the incubation with only the free fatty acid (FFA) (Freitas et al., 2020).

Additionally, to evaluate whether the increase in myoblasts viability occurred due to Ut extract presence in the culture medium during incubation with PA, or if compounds present in the Ut extract might have participated on the cellular response against PA cytotoxicity, cultures were washed with PBS between the pretreatment with the Ut extract and the incubation with the FFA. In this preventive condition, it was observed an increase of 45% in cell viability, suggesting that compounds in Ut extract might have entered the cell (Ying et al., 2018) protecting some cellular organelles and/or have activated some intracellular antioxidant defense pathway. As shown by Duchnowicz et al. (2021), compounds from the Ut bark extract not only penetrate deeper layers of cell membranes of erythrocytes, but are transferred inside the cell.

Such effects of Ut bark extracts compounds were also described in Tarahovsky et al. (2014), which suggested that secondary metabolites are able to penetrate the hydrophobic site of the lipid bilayer, particularly into the compartments known as lipid raft and increase the antioxidative potential, facilitating the protection of membrane lipids against oxidation.

Based on this evidence, we also evaluated the effect of Ut extract in the recovery of cell viability, followed by the incubation of cultures with 500  $\mu$ M PA, which was called therapeutic treatment (Kim et al., 2018). In this condition, it was observed an increase up to 70% in cell viability, suggesting that 250  $\mu$ g/ml Ut extract act against palmitate-induced lipotoxicity on myoblasts. Particularly, the treatment with Ut aqueous extract for 6 h was more effective for cell viability recovery, which could suggest its use even after cells have been damaged by the FFA action. Also, the Ut aqueous extract treatment improved cell metabolism in a time-dependent manner.

The treatment with Ut aqueous extract for 2 or 6 h, in both preventive or therapeutic conditions, resulted in reduction of ROS generation when compared to the cultures incubated with only 500  $\mu$ M PA, demonstrating that the treatment with Ut extract for 6 h was more efficient in comparison to the period of 2 h. Interestingly, the treatment with Ut extract for 2 and 6 h in cultures incubated with 100  $\mu$ M PA, considered a physiological concentration (Freitas et al., 2020), resulted in an increase on ROS production, compared to control, while the Ut extract promoted a decrease in the amount of ROS in cells incubated with 500  $\mu$ M PA. This dual response could be due to the variety of secondary metabolites present in Ut aqueous extract, as reported by Heitzman et al. (2005) and Ciani et al. (2018). According to Calabrese & Mattson (2017), this effect may be related to the biphasic response of cells or organisms to the increasing exposure to an agent, exhibiting an inhibitory or beneficial effect, which can be understood as an adaptive response to different stimuli, known as hormesis effect.

Indeed, we showed that both preventive and therapeutic conditions with the Ut aqueous extract were efficient for cell viability recovery, promoting a decrease in the percentage of cells with fragmented DNA

and on high production of ROS in cells exposed to 500  $\mu$ M PA.

The Ut effect on skeletal muscle cells exposed to PA has been attributed to its antioxidant content, which can react with the ROS and other free radicals, neutralizing them and preventing oxidative damage to membrane lipids (Heitzman et al., 2005; Lehninger et al., 2014, Borçari et al., 2020). Specifically, Abouelela et al. (2020) investigated the antioxidant effect of the alkaloid cinchonain, isolated from the aqueous extract of *Ceiba pentandra* plant and tested in adult rats with renal deficiency. They found in assays for ROS quantification and immunohistochemistry analysis that this compound was able to increase hepatocytes viability, which was correlated to its antioxidant potential.

In summary, the presented results might contribute to a better understanding of the molecular mechanism involved in the Ut aqueous extract action on palmitate-induced lipotoxicity, in skeletal muscle tissue, opening perspectives for the therapies based on the redox state reestablishment using herbal formulations with antioxidants.

## 5. CONCLUSIONS

The viability of myoblasts incubated with PA, from concentration of 250  $\mu$ M, was significantly reduced, confirming the lipotoxic effect of this FFA, whereas the Ut aqueous extract promoted increase in viability of C2C12 skeletal muscle cells.

Also, cells pretreatment with Ut aqueous extract, for 2 or 6 h, whether in the preventive or therapeutic conditions, resulted in increase in viability, decreased DNA fragmentation and reduced ROS production in muscle cells exposed to 500  $\mu$ M PA, which consists of a cytotoxic concentration.

The observed effects on muscle cells exposed to PA suggest the involvement of the FFA in pathways related to the oxidative stress.

## Declarations

### ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental protocols were approved by Ethical Committee of University of Sao Paulo, and methods were carried out in accordance with institutional procedures and world guidelines and regulations.

### CONSENT FOR PUBLICATION

Not applicable.

### DATA AVAILABILITY

Previously reported data were used to support this study and are available at DOI 10.22533/at.ed.40420240616. The previous study of our group is cited as the reference Freitas *et al.*

(2020). Data should be request to vanunes@ib.usp.br.

## AUTHOR CONTRIBUTIONS

All authors participated in the writing and reviewing of the manuscript.

## COMPETING INTERESTS

We declare that there is no financial or other potential conflict of interest.

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## Figures

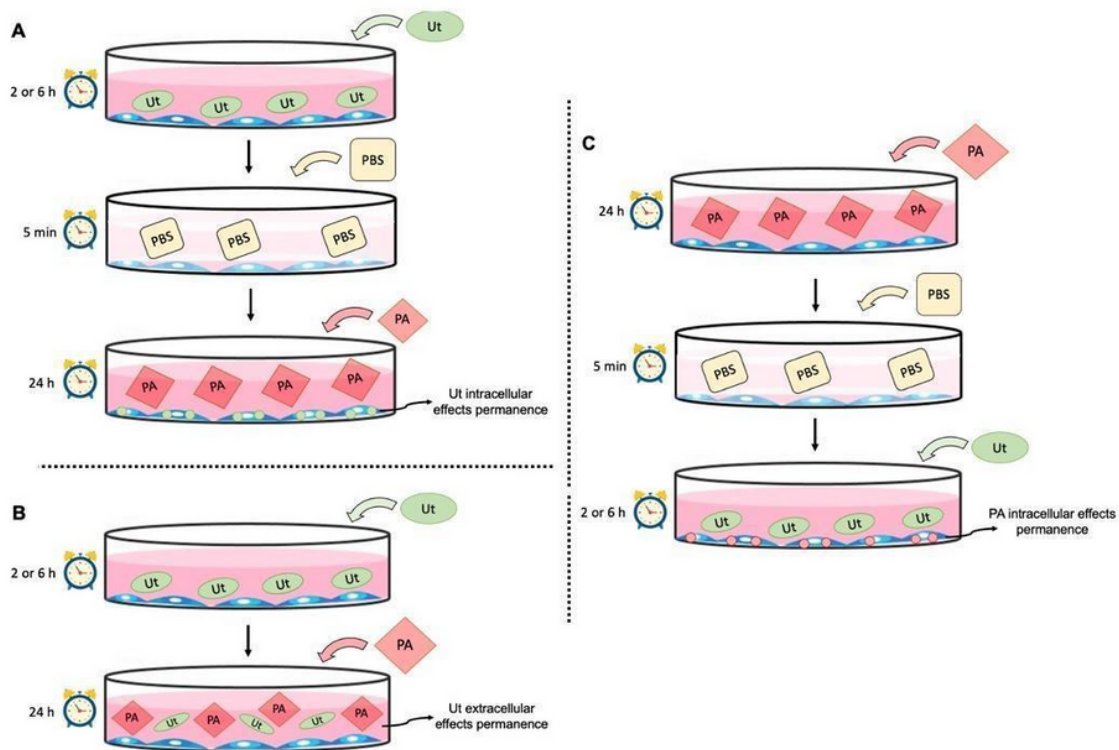


**Figure 1**

**Schematic experimental approaches.** (A) Cells were pretreated, for 2 or 6 h, with *Uncaria tomentosa* (Ut) aqueous extract; after these periods, cultures were washed with phosphate buffer saline (PBS) before incubation with the palmitate (PA) solution in different concentrations, for 24 h ("preventive" condition); (B) Cells were pretreated, for 2 or 6 h, with Ut aqueous extract and cultures were incubated with PA in different concentrations, for 24 h. (C) Cells were incubated with PA in different concentrations, for 24 h; after, cultures were washed with PBS and, then, Ut aqueous extract was added, for 2 or 6 h ("therapeutic" condition).

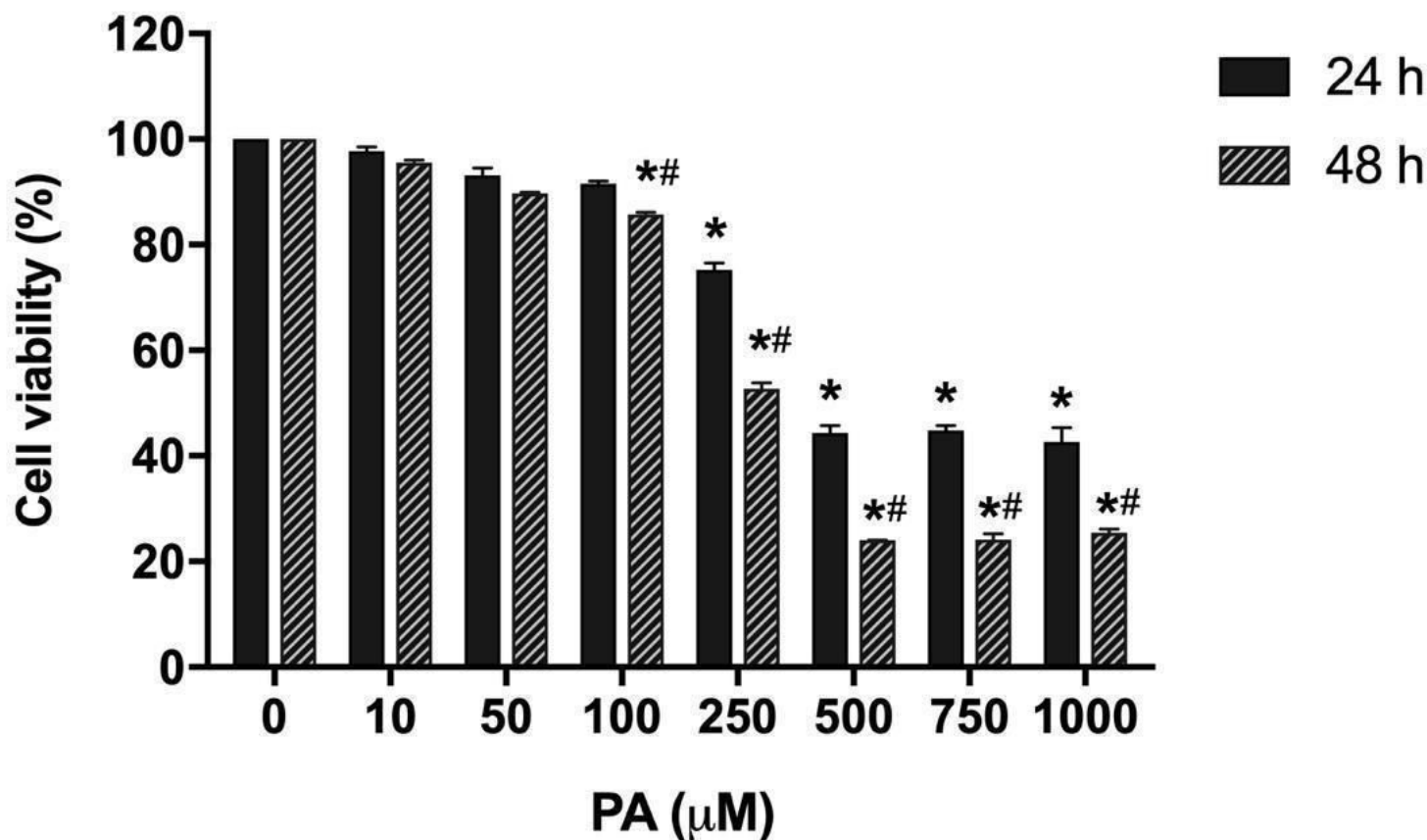


Figure 2

**Effect of palmitate (PA) on C2C12 myoblasts viability.** Cells were incubated for 24 or 48 h, with PA, at concentrations from 10 to 1000 μM and evaluated by the MTT method. The data are presented as the mean  $\pm$  SE of four individual experiments in triplicates. The values are expressed as the percentage in relation to the control. \* Indicates difference compared to the control without the FFA; # indicates difference between the two periods of time ( $p < 0.05$ ).

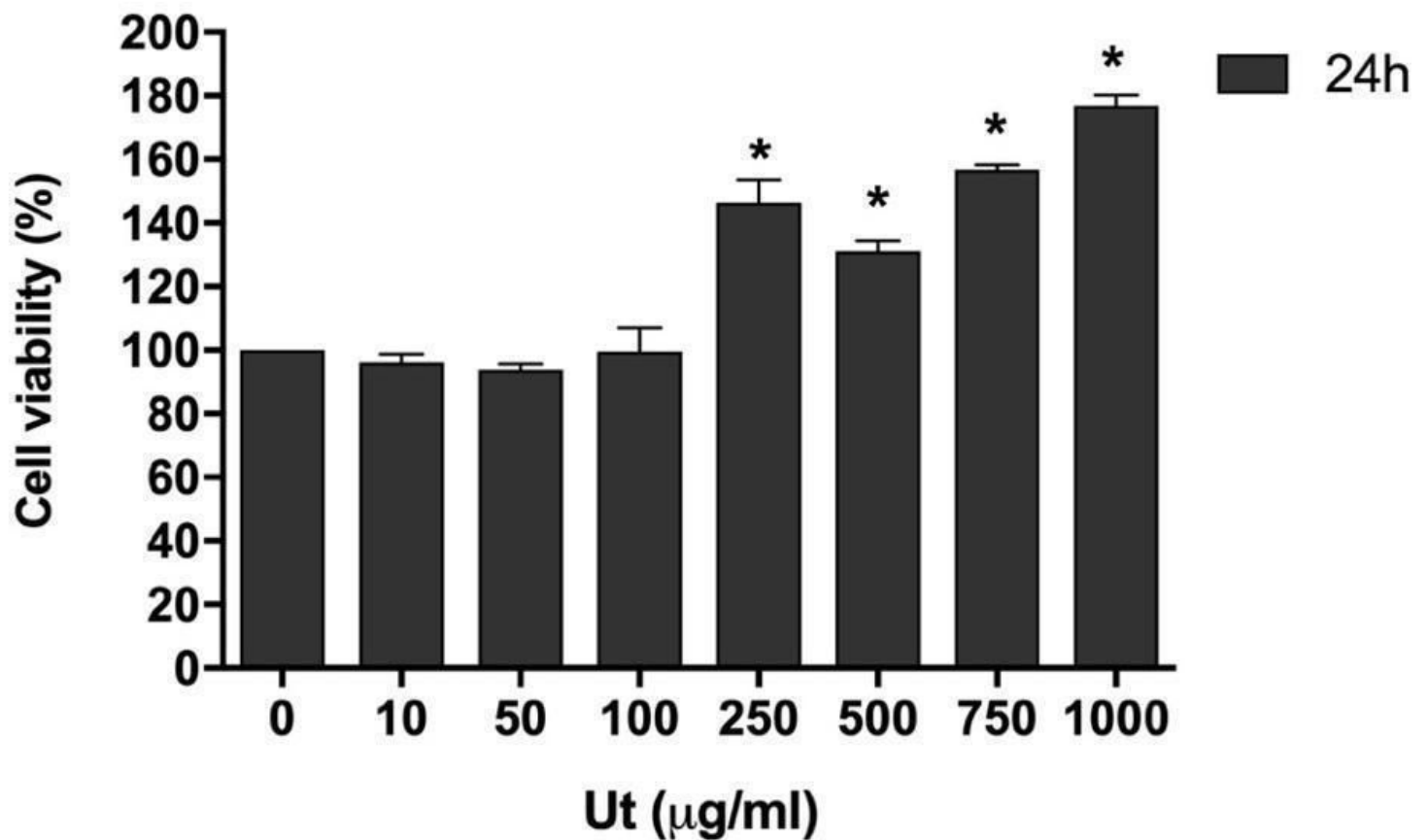
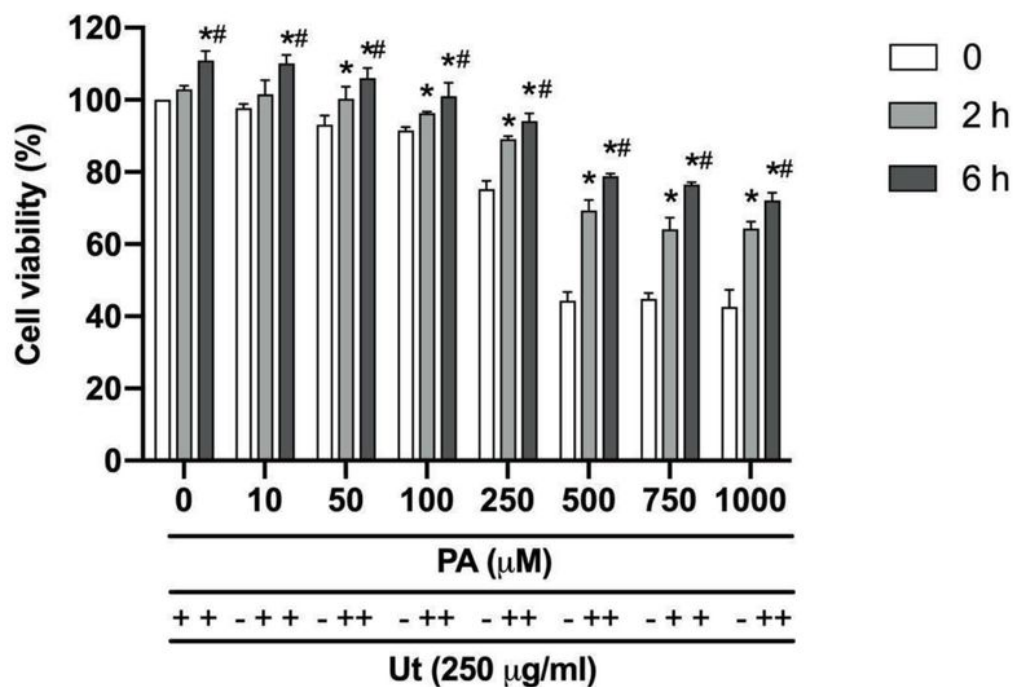


Figure 3

**Effect of the *Uncaria tomentosa* (Ut) aqueous extract on the viability of C2C12 myoblasts.** Cells were incubated for 24 h with the Ut aqueous extract (10, 50, 100, 250, 500, 750 and 1000 µg/ml) and evaluated by the MTT method. The data are presented as the mean  $\pm$  SE of four individual experiments in triplicates. The values are expressed as a percentage in relation to the control. \* Indicates difference in comparison to control without the Ut extract ( $p < 0.05$ ).



B)

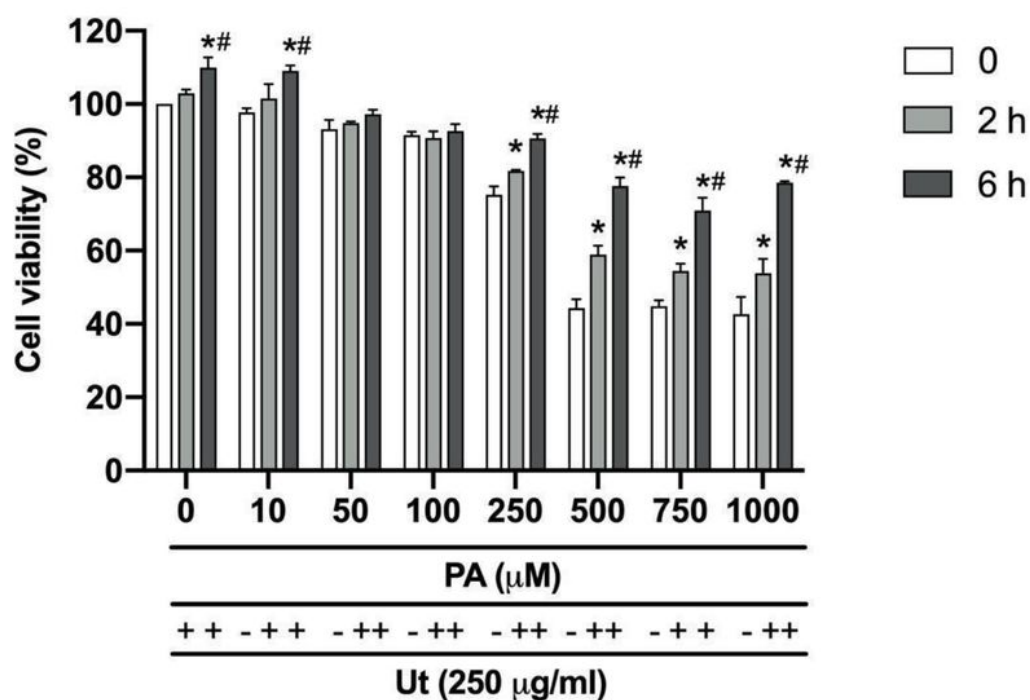


Figure 4

**Effect of the Ut aqueous extract against the toxicity of PA in C2C12 myoblasts cells.** A) The cultures were pretreated with 250 μg/ml Ut extract, for 2 or 6 h, and then incubated with PA for 24 h. B) Cultures were pretreated with the 250 μg/ml Ut extract, for 2 or 6 h, washed with PBS and, subsequently, incubated with PA, for 24 h. Cell viability was assessed using the MTT method. The data are presented as the mean ± SE of four individual experiments in triplicates and the values are expressed as a percentage, in relation to

the control with only FFA. \* Indicates difference in comparison to control of each group; # indicates the difference between pretreatment with Ut for 2 or 6 h ( $p<0.05$ ).

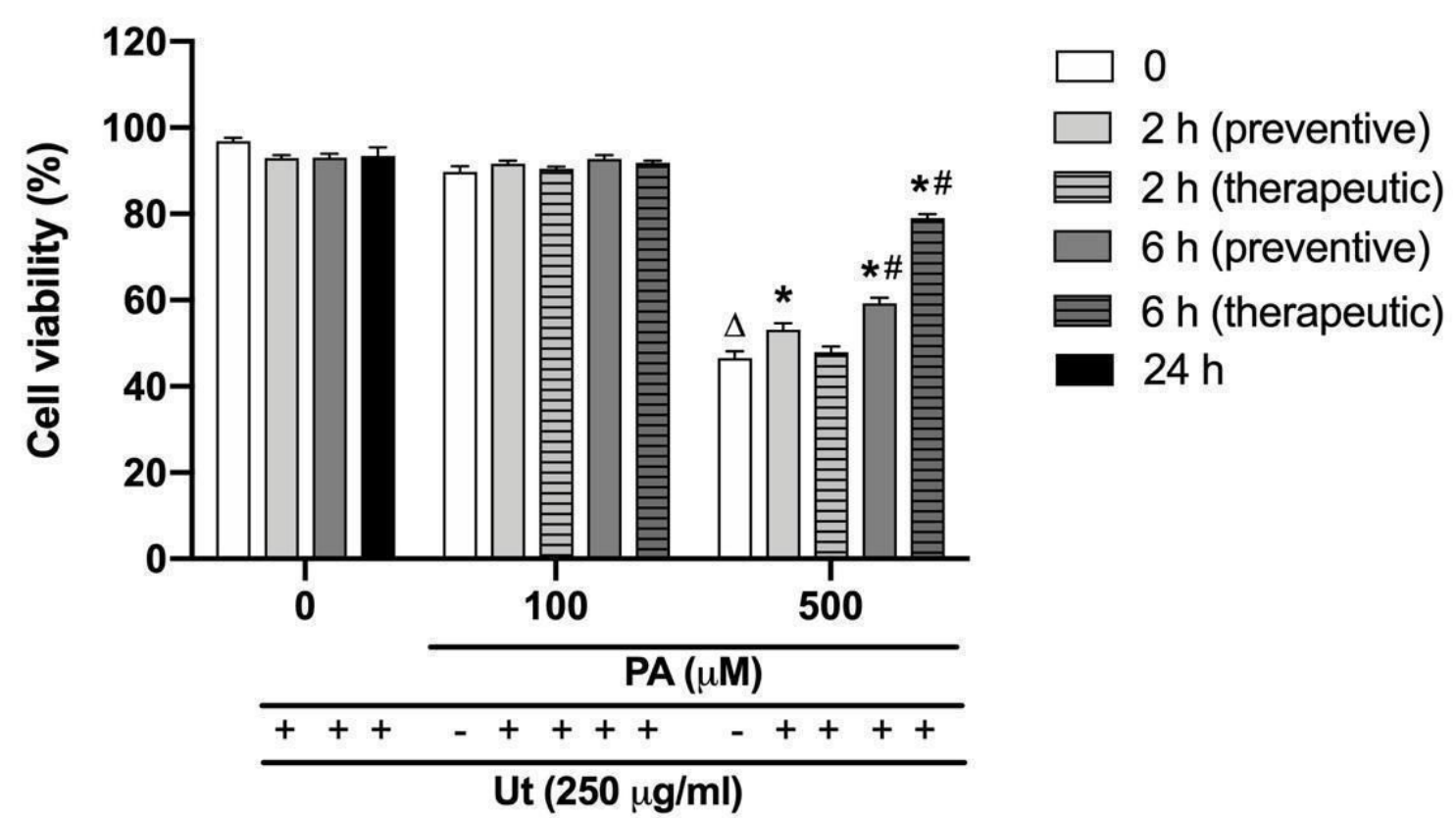


Figure 5

**Effect of the Ut aqueous extract against the toxicity of PA in C2C12 myoblasts.** Cells were treated with the 250  $\mu\text{g/ml}$  Ut aqueous extract, for 2 or 6 h, and incubated with 100 or 500  $\mu\text{M}$  PA, for 24 h. Cell viability was assessed by flow cytometry using propidium iodide. 10.000 events were acquired per sample. The data are presented as the mean  $\pm$  SE of three individual experiments in duplicates and the values are expressed as the percentage in relation to the control. \* Indicates difference compared to control with only the FFA; # indicates difference between treatments with Ut extract for 2 or 6 h;  $\Delta$  indicates a difference from the control (cultures incubated with only ethanol) ( $p<0.05$ ).

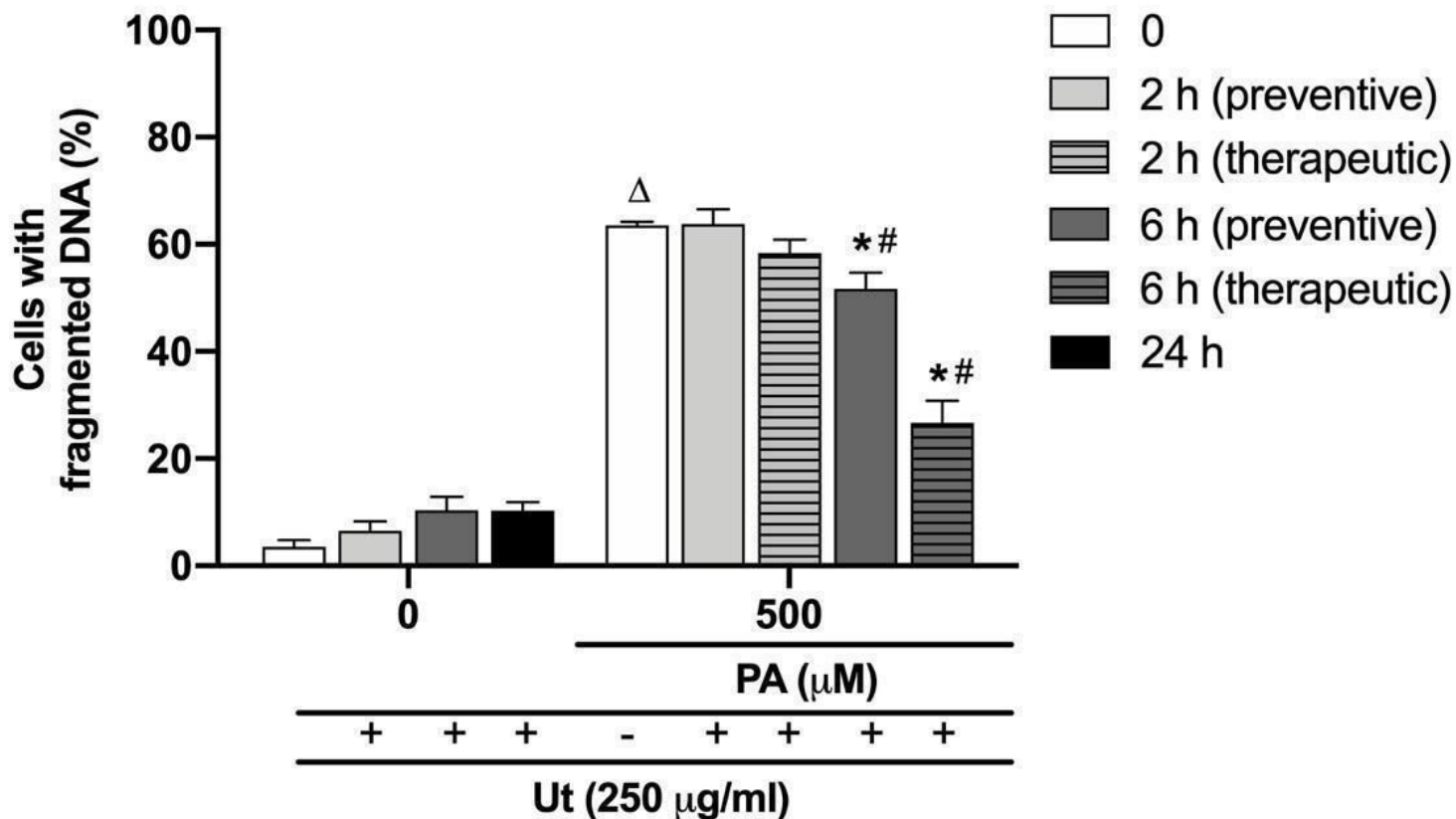


**Figure 6**

**Effect of the Ut aqueous extract on palmitate-induced DNA fragmentation in C2C12 myoblasts.** The cells were treated with the 250 μg/ml Ut extract, for 2 or 6 h, and incubated with 100 or 500 μM PA, for 24 h. 10.000 events were acquired per sample. The data are presented as the mean ± SE of three individual experiments in duplicates and the values are expressed as the percentage in relation to the control, according to the number of viable cells in each sample. \* Indicates difference in relation to the control with only the FFA; # indicates the difference between treatments with Ut for 2 or 6 h; Δ indicates difference in relation to the cultures incubated with only ethanol (p<0.05).

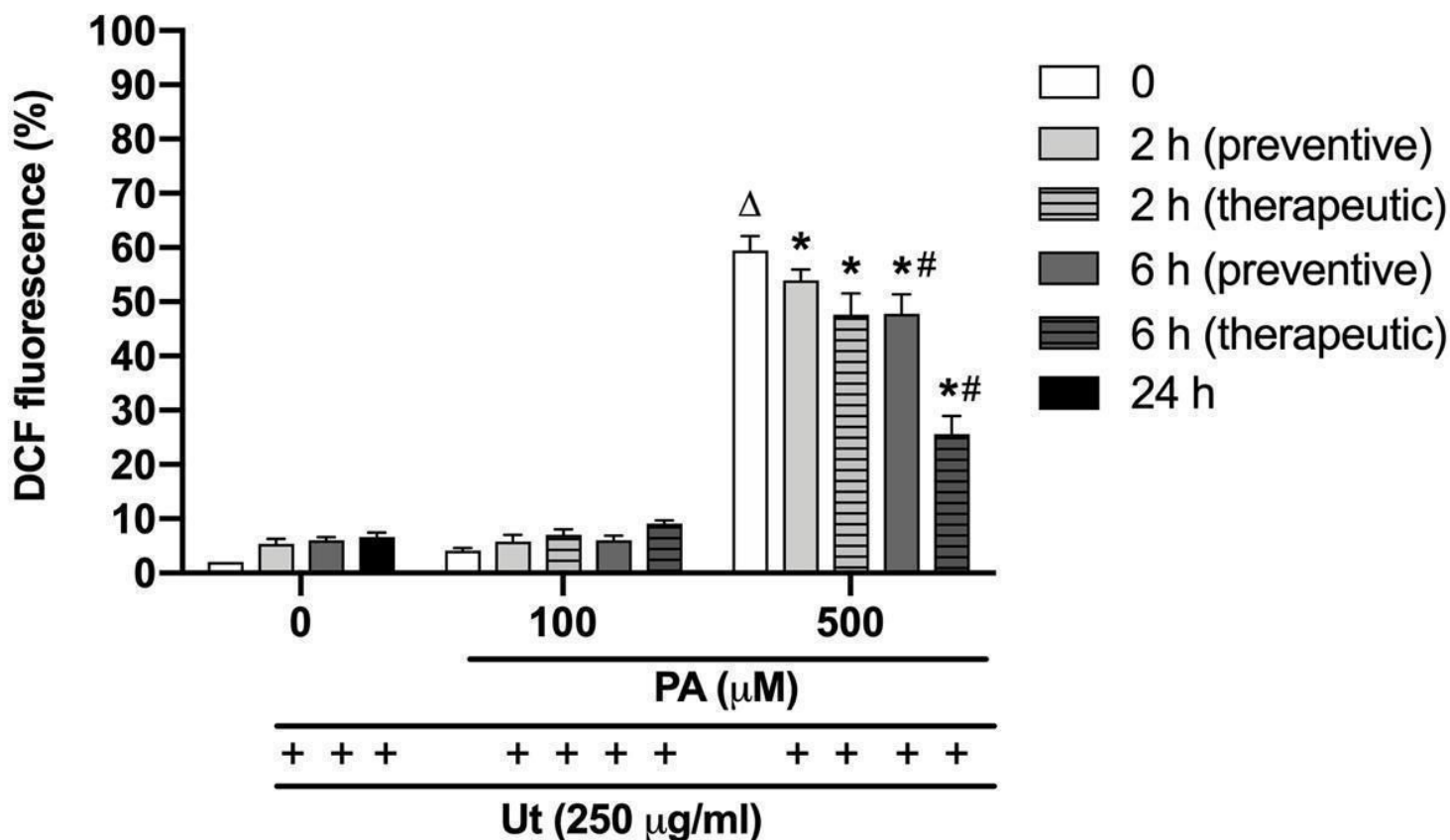


Figure 7

**Determination of ROS production in C2C12 myoblasts incubated with PA, in the absence or presence of Ut extract.** The samples were evaluated by fluorescence spectroscopy, at  $\lambda_{\text{excitation}} = 485 \text{ nm}$  and  $\lambda_{\text{emission}} = 528 \text{ nm}$ . The ROS formation was measured in fluorescence arbitrary units (FAU) and expressed as the percentage considering the number of viable cells in each sample, in relation to the control. The data are presented as the mean  $\pm$  SE of three individual experiments in triplicates. \* Indicates difference in relation to the control with only the FFA; # indicates difference between treatments with Ut extract for 2 or 6 h;  $\Delta$  indicates difference in relation to cultures incubated with only ethanol ( $p < 0.05$ ).

## Supplementary Files

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