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EPICATECHIN AS A MODULATOR OF MITOCHONDRIAL FUNCTION IN THP-1 LINEAGE MONOCYTES[1]

EPICATEQUINA COMO MODULADOR DA FUNÇÃO MITOCONDRIAL EM MONÓCITOS DA LINHAGEM THP-1

Djenifer Leticia Ulrich Bick[2], Gabriela Geraldo Sangoi[3], Carolina Bordin Davidson[3], Taíse Regina Schuster Montagner[2], Amanda Kolinski Machado[2], Aline Ferreira Ourique[3] e Alencar Kolinski Machado[3]

ABSTRACT

Public health problems are increasingly prevalent worldwide, and many diseases have a multifactorial nature and complex pathophysiology. In this context, mitochondrial dysfunction has emerged as a recent target of investigation, being observed in several conditions such as neurodegenerative and metabolic diseases, highlighting the complexity of their treatment. Given this scenario, the search for new therapeutic alternatives that act at the mitochondrial level represents an important focus of research. Epicatechin (EC), a flavonoid found in various plants, possesses well-known beneficial biological properties. This study aims to analyze the effects of EC in reversing mitochondrial damage induced by known oxidative agents. THP-1 monocyte cell lines were exposed to rotenone, sodium azide, or sodium nitroprusside and subsequently treated with epicatechin. Assays were performed to evaluate cell viability, reactive oxygen species (ROS) production, nitric oxide release, the presence of extracellular double-stranded DNA (dsDNA), and DNA damage through the alkaline comet assay. The results are consistent with the literature and indicate that, at certain concentrations, EC can attenuate the induced damage in the evaluated parameters, demonstrating potential as a cellular protective agent. Thus, EC shows the ability to protect cells and minimize mitochondrial damage, highlighting its promise as a therapeutic candidate for further investigation.

Keywords: antioxidants; mitochondrial dysfunction; oxidative stress.

RESUMO

Os problemas de saúde pública são cada vez mais prevalentes globalmente e muitas doenças apresentam caráter multifatorial e fisiopatologia complexa. Nesse contexto, a disfunção mitocondrial representa um alvo recente de estudos, sendo observada em diversas patologias como doenças neurodegenerativas e metabólicas, demonstrando complexidade nos tratamentos. Diante disso, a busca por novas alternativas terapêuticas que atuem sobre danos a nível mitocondrial representa um alvo importante nas pesquisas. A epicatequina (EC), um flavonoide presente em diversas plantas, possui propriedades biológicas benéficas conhecidas. Esse estudo busca analisar a ação da EC na reversão dos danos mitocondriais induzidos por agentes oxidantes conhecidos. Monócitos de linhagem THP-1 foram expostos a rotenona, azida sódica ou nitroprussiato de sódio e em seguida foram tratados com a epicatequina. Foram realizados ensaios para avaliar a viabilidade celular, produção de espécies reativas de oxigênio, liberação de óxido nítrico, presença de DNA de cadeia

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2 Biomedicine Course, Franciscan University (UFN), Santa Maria, RS, Brazil. E-mail: djenifer.ulrich@ufn.edu.br; taise.schuster@ufn.edu.br; amanda.kolinski@ufn.edu.br. ORCID: https://orcid.org/0009-0002-1434-8297; https://orcid.org/0009-0004-1985-2243; https://orcid.org/0009-0004-3758-8773

3 Graduate Program in Nanosciences, Franciscan University (UFN), Santa Maria, RS. E-mail: gabriela.sangoi@ufn.edu.br; carolinabd99@gmail.com; aline.ourique@ufn.edu.br; alencar.machado@ufn.edu.br. ORCID: https://orcid.org/0009-0008-0069-458X; https://orcid.org/0000-0003-4956-2787; https://orcid.org/0000-0003-0828-774X; https://orcid.org/0000-0003-2003-8420

dupla (dsDNA) no meio extracelular e os danos ao DNA por meio do ensaio cometa alcalino. Os resultados corroboram com a literatura e indicam que, em determinadas concentrações, a EC é capaz de atenuar os danos induzidos nos fatores pesquisados se mostrando como um potencial agente de proteção celular. Dessa forma, verifica-se que a EC é capaz de proteger as células e minimizar o dano mitocondrial se mostrando como um potencial agente a ser pesquisado.

Palavras-chave: *antioxidantes, disfunção da mitocôndria, estresse oxidativo.*

INTRODUCTION

Public health problems have been intensifying globally, becoming issues of significant social and economic relevance. According to the World Health Organization (WHO), non-communicable chronic diseases (NCDs) account for approximately 74% of deaths worldwide. These pathologies often have a multifactorial nature and a complex pathophysiology that is not yet fully understood, making it challenging to develop pharmacological treatments that are both effective and have minimal side effects (Wehrmeister; Wenddt; Sardinha, 2022).

In this context, diseases involving mitochondrial dysfunction have emerged as relevant targets in human disease research. Understanding the pathophysiological mechanisms underlying these disorders often necessitates an individualized or disease-specific approach. Mitochondrial damage has been identified in various metabolic, neuropsychiatric, and neoplastic diseases (Disha *et al.,* 2024). The mitochondrion is an organelle involved in several biological processes, including the production of approximately 90% of adenosine triphosphate (ATP)- a key molecule in maintaining cellular homeostasis - through coordinated oxidative phosphorylation, cholesterol metabolism, cell signaling, and inflammatory pathways (Sharma; Sampath, 2019; Marchi *et al.,* 2023).

However, when cellular homeostasis is disrupted, mitochondria-induced inflammatory responses can contribute to the etiology of human disorders (Marchi *et al*., 2024). Studies have shown that mitochondrial dysfunction leads to an imbalance in oxidative metabolism, triggering the inflammatory cascade and increasing the production of reactive oxygen species (ROS) (Andreazza *et al*., 2010). Additionally, several mitochondrial components and metabolic products can function as Damage-Associated Molecular Patterns (DAMPs) and, depending on other factors, may trigger cell death when homeostatic capacity is lost (Jomova *et al.,* 2023; Land, 2023). Under adverse conditions, cells activate a broad range of mechanisms to restore proper function and repair molecular damage, including mitochondrial responses, which coordinate and regulate cellular stress and damage responses (Ylikallio; Suomalainen, 2012). Exposure to DAMPs can activate immune pathways such as type I interferon release, mitophagy, and activation of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome (Nakahira *et al*., 2011).

In this context, there is a growing interest in identifying strategies to reduce the damage caused by mitochondrial deficiencies. Therefore, natural products have emerged as promising

therapeutic alternatives to mitigate the effects of such alterations. Studies have shown that epicatechin (EC), a flavonoid found in various plants such as cocoa, green tea, and açaí, has been associated with improvements in mitochondrial function (Dorneles *et al.,* 2021; Qu *et al*., 2021). EC is recognized for its antioxidant and anti-inflammatory properties (Davidson *et al.,* 2024), as well as for its neuroprotective, cardiovascular, and metabolic benefits (Jideani *et al.,* 2021). In addition, antioxidants act by reducing the levels of ROS and other reactive species that cause harmful effects; due to their redox properties, they also tend to prevent the formation of toxic and mutagenic products (Jideani *et al*., 2021).

Supplementation with EC affects multiple mitochondrial functions, including the respiratory cycle, by increasing the levels of structural proteins and of complexes I and II of the electron transport chain (Moreno-Ulloa *et al*., 2018). Furthermore, both in vivo and in vitro models have demonstrated that EC supplementation significantly impacts the content of respiratory chain complexes in different parts of the body, as well as the lipid composition of the mitochondrial membrane (Moreno-Ulloa *et al.,* 2015). Collectively, these findings support the understanding that EC can acutely modulate mitochondrial respiration through direct effects on the complexes and possibly other components of the respiratory chain (Daussin; Heyman; Burelle, 2021). Thus, the objective of this study was to analyze the potential of EC to attenuate mitochondrial dysfunction using different in vitro models of cellular stress exposure.

METHODS

EXPERIMENTAL DESIGN

In this study, an in vitro experimental approach was employed to investigate the effects of epicatechin (EC) on reversing and mitigating mitochondrial dysfunction induced by well-known stressor agents: rotenone, sodium azide, and sodium nitroprusside. For this purpose, monocytes were initially cultured and subsequently exposed to the stressors to assess the damage profile. Following exposure, the cells were treated with commercially obtained EC. All experiments were conducted in at least triplicate.

CELL CULTURE AND TREATMENTS

The human monocytic cell line THP-1 was selected for this study due to its widespread use as a model for investigating inflammatory responses, cellular protection mechanisms, and mitochondrial activity (Chanput; Mes; Wichers, 2014; Kwok; Kwak; Andreazza, 2023). The cell line was commercially obtained from the Rio de Janeiro Cell Bank (BCRJ) and cultured under appropriate conditions.

The culture medium used was modified RPMI 1640, containing 2 mM L-glutamine, 4500 mg/L glucose, and 10 nM HEPES, supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, São Paulo, Brazil) and 1% (v/v) antibiotics (streptomycin and penicillin - Sigma-Aldrich, São Paulo, SP, Brazil). Cells were maintained in culture under controlled conditions, in a 5% CO_2 atmosphere at 37 °C, until reaching the ideal confluency for experimental procedures.

Subsequently, cells were seeded into round-bottom 96-well plates at a concentration of 2.5×10^5 cells/mL per well and treated with known oxidative agents. Concentration curves were established to identify the most effective concentrations in reducing cell viability, reflecting induced mitochondrial dysfunction. The stressor agents used were: (I) rotenone (5, 15, 30, 60, 100, 150, and 200 nM) (Kim *et al.,* 2015); (II) sodium azide (1, 5, 10, 20, 40, 80, and 160 µg/mL) (Zuo *et al.,* 2019); and (III) sodium nitroprusside (1, 3, 10, 30, and 100 µM) (Li *et al*., 2018). For rotenone and sodium azide, the incubation period was 24 hours. For sodium nitroprusside, a 48-hour incubation was used, as no significant changes were observed after 24 hours of exposure. Following this, assays were performed using EC in a concentration curve (0.01, 0.05, 0.1, 1, 10, and 100 µg/mL) on cells previously exposed to the stressors, to evaluate the bioactive molecule's ability to reverse cellular damage. Dilutions of both the pro-oxidant agents and EC were prepared using RPMI 1640 culture medium, which served as the vehicle, ensuring proper dispersion and contact with the cells in the plates.

CELL VIABILITY AND PROLIFERATION ASSAY

The MTT assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; Sigma-Aldrich, São Paulo, Brazil) was used as a colorimetric assay to assess cell viability (after 24 hours of incubation) and cell proliferation (after 48 and 72 hours, considering the cell doubling time), following the methodology described by Kang *et al*. (2010). After treatments, cells were incubated with MTT for 1 hour in a 5% CO_2 incubator at 37°C. Metabolically active cells reduce MTT, a yellow tetrazolium salt, into purple formazan crystals via mitochondrial dehydrogenase enzyme activity. After incubation, the formed crystals were solubilized by adding dimethyl sulfoxide (DMSO). Optical density in each well was measured spectrophotometrically. Absorbance was read at 570 nm using a Synergy microplate reader (Biotek, Santa Clara, CA, USA).

NITRIC OXIDE LEVEL MEASUREMENT

The indirect assay for nitric oxide (NO) levels was conducted according to Choi *et al*. (2012), based on the use of the Griess reagent, which enables the detection of metabolic nitrite (NO_2^-) and nitrate (NO_3^-) in the samples-an important parameter for assessing anti-inflammatory activity. For the assay, 50 µL of sample supernatant and 50 µL of Griess reagent were added to each well of a 96-well

plate and incubated for 15 minutes at room temperature protected from light. The presence of these molecules produces a pink coloration in the wells, and the colorimetric reading was performed at 540 nm using a Synergy microplate reader (Biotek, Santa Clara, CA, USA).

TOTAL REACTIVE OXYGEN SPECIES MEASUREMENT

A semi-quantitative analysis of the total ROS rate was conducted using a fluorometric assay based on the reagent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Costa *et al.,* 2012). This test relies on the ability of this reagent to be deacetylated by cytosolic esterases into dichlorodihydrofluorescein (DCFH), which, upon contact with ROS, is metabolized into dichlorofluorescein (DCF), a compound capable of emitting fluorescence. For the assay, 50 µL of supernatant sample, 65 µL of Tris-HCl, and 10 µL of DCFH-DA were used. The 96-well plate was incubated for 1 hour protected from direct light at room temperature. The emitted fluorescence was detected at an emission wavelength of 528 nm with excitation at 485 nm using a Synergy microplate reader (Biotek, Santa Clara, CA, USA).

EXTRACELLULAR dsDNA QUANTIFICATION ASSAY

The assay used to quantify extracellular double-stranded DNA (dsDNA) resulting from cell apoptosis was performed using the Quanti-iT™ PicoGreen® dsDNA kit (Invitrogen®), which binds to free dsDNA and emits fluorescence (Cadoná et al., 2014). For the assay, 10 µL of cell supernatant, 80 µL of Tris-EDTA (TE) buffer, and 10 µL of PicoGreen® solution were combined. After incubation for 5 minutes at room temperature protected from light, fluorescence intensity was measured with an excitation wavelength of 485 nm and emission at 528 nm using a Synergy microplate reader (Biotek, Santa Clara, CA, USA).

DNA DAMAGE DETECTION ASSAY

To evaluate potential DNA damage, the alkaline comet assay was performed. This test was conducted using THP-1 cells at a concentration of 3×10^5 cells/mL, treated for 24 hours with the established EC concentration curve, following the protocol described by Singh *et al.* (1988). The alkaline comet assay is an electrophoresis-based technique used to detect single-strand DNA breaks (ssDNA), allowing the assessment of DNA damage and repair at the individual cell level. This assay is widely applied in cellular toxicology and in vitro genotoxicity studies.

Cells were treated with EC and, after 24 hours of incubation, collected and resuspended in phosphate-buffered saline (PBS). An aliquot of 20 ***µ***L of the cell suspension was mixed with 0.75%

low-melting-point agarose and immediately spread onto glass slides pre-coated with a layer of 1.5% normal agarose. After solidification, the slides were immersed in a lysis solution containing Triton X-100 and NaCl for 24 hours at 4°C in a dark container to remove cellular components and expose the DNA. Subsequently, the slides were incubated for 1 hour in an alkaline solution (pH > 13) to denature DNA strands, followed by electrophoresis in alkaline buffer at 25 V for 20 minutes. After electrophoresis, the slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with silver nitrate in a water bath at 37°C for approximately 30 minutes, and then immersed in a stop solution (1% acetic acid). The slides were air-dried overnight and later analyzed by conventional light microscopy for evaluation of DNA strand breaks.

The extent of the comet tail represents the migration of DNA fragments on the slide and is proportional to the degree of DNA fragmentation, predominantly reflecting ssDNA breaks, which are evidenced by the migration of genetic material during alkaline electrophoresis. Analysis was performed manually by two independent evaluators by counting a total of 100 nuclei per slide. Slides were prepared in duplicate for each EC concentration and controls. The results are expressed as DNA damage indices.

STATISTICAL ANALYSIS

Fluorescence and absorbance readings were performed using the Synergy® H1 microplate reader (Biotek, Santa Clara, CA, USA). After obtaining raw data, GraphPad Prism® software (GraphPad Prism®, 2018; San Diego, CA, USA) was used for graph construction and statistical analysis, employing one-way ANOVA followed by Tukey's post hoc test. Results with $p < 0.05$ were considered statistically significant.

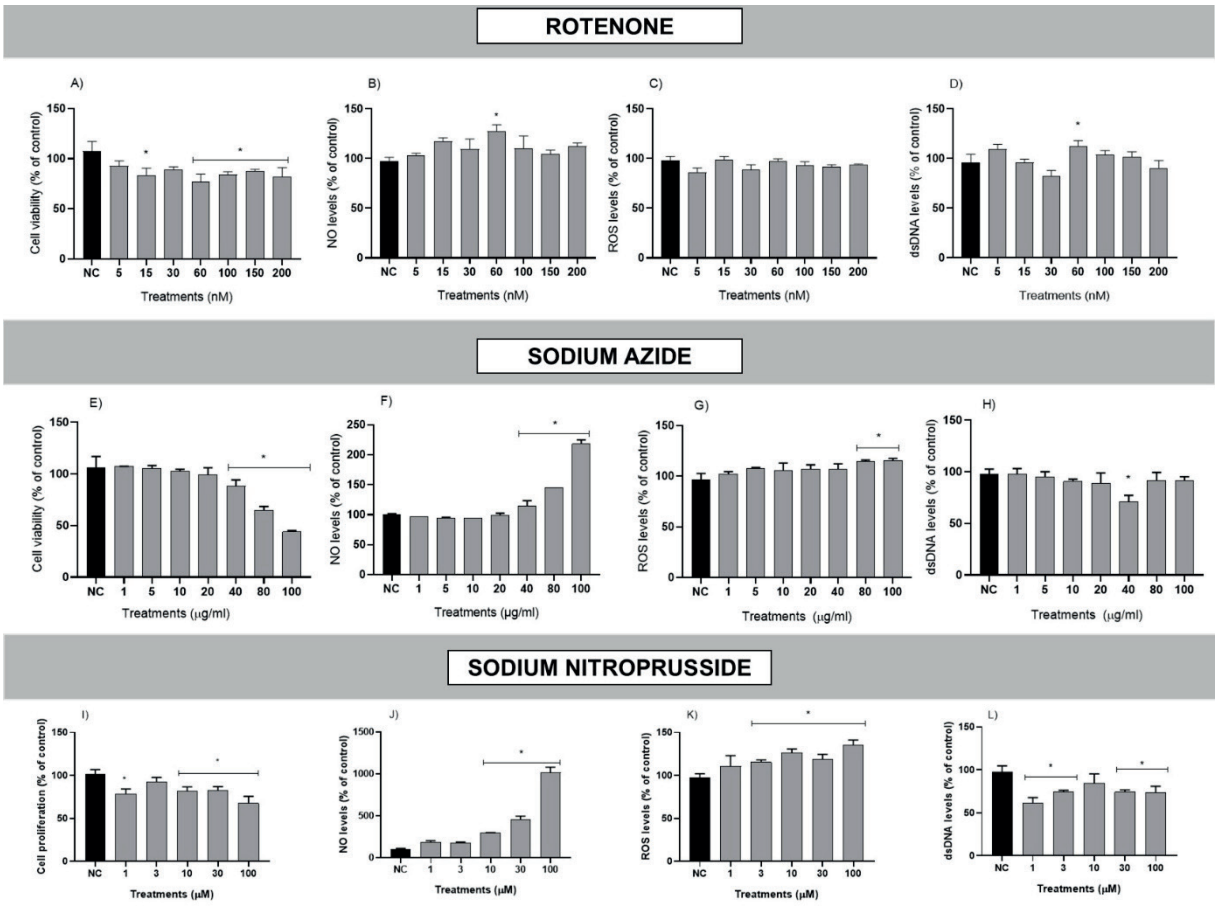
RESULTS AND DISCUSSION

The results obtained from the *in vitro* assays are described below. THP-1 cells exposed to different concentrations of rotenone for 24 hours exhibited a significant reduction in cell viability at all concentrations except 30 nM, compared to the negative control (NC). These findings are consistent with those reported by Currò *et al*. (2022), who also observed reduced viability when using the same stressor agent. Analysis of nitric oxide (NO) and extracellular dsDNA showed increased levels only at the 60 nM rotenone concentration compared to the NC. Additionally, no increase in reactive oxygen species (ROS) production was observed after 24 hours of THP-1 exposure to rotenone, which aligns with results reported by Hu and Zhu (2007) during the same incubation period. Considering the overall data, the rotenone concentration chosen for subsequent assays was 60 nM.

Regarding sodium azide, a reduction in cell viability and an increase in NO release were observed after 24 hours of treatment in cells exposed to concentrations ranging from 40 to 100 ***μ***g/mL compared to the NC. An increase in ROS release was also noted, consistent with findings by Zhang *et al*. (2021), where elevated ROS levels were observed under similar conditions. The concentration of 40 ***μ***g/mL was selected for subsequent experiments due to its representative damaging effect.

Furthermore, the third stressor agent used was sodium nitroprusside, a pro-oxidant known for its nitric oxide (NO) donating properties and its role as an inhibitor of complex IV in the respiratory chain (Feelisch, 1998). The results showed a reduction in cell proliferation at all concentrations except 3 ***μ***M when compared to the negative control (NC) after 48 hours of exposure. Increases in NO and reactive oxygen species (ROS) were also observed at certain concentrations, consistent with data reported by Cardaci *et al.* (2008) under similar conditions. Considering the damage induced, the concentration of 30 ***μ***M was selected for subsequent experiments. The results of the assays with rotenone, sodium azide, and sodium nitroprusside are shown in Figure 1.

Figure 1 - Concentration curve of stressor agents in THP-1 cells.

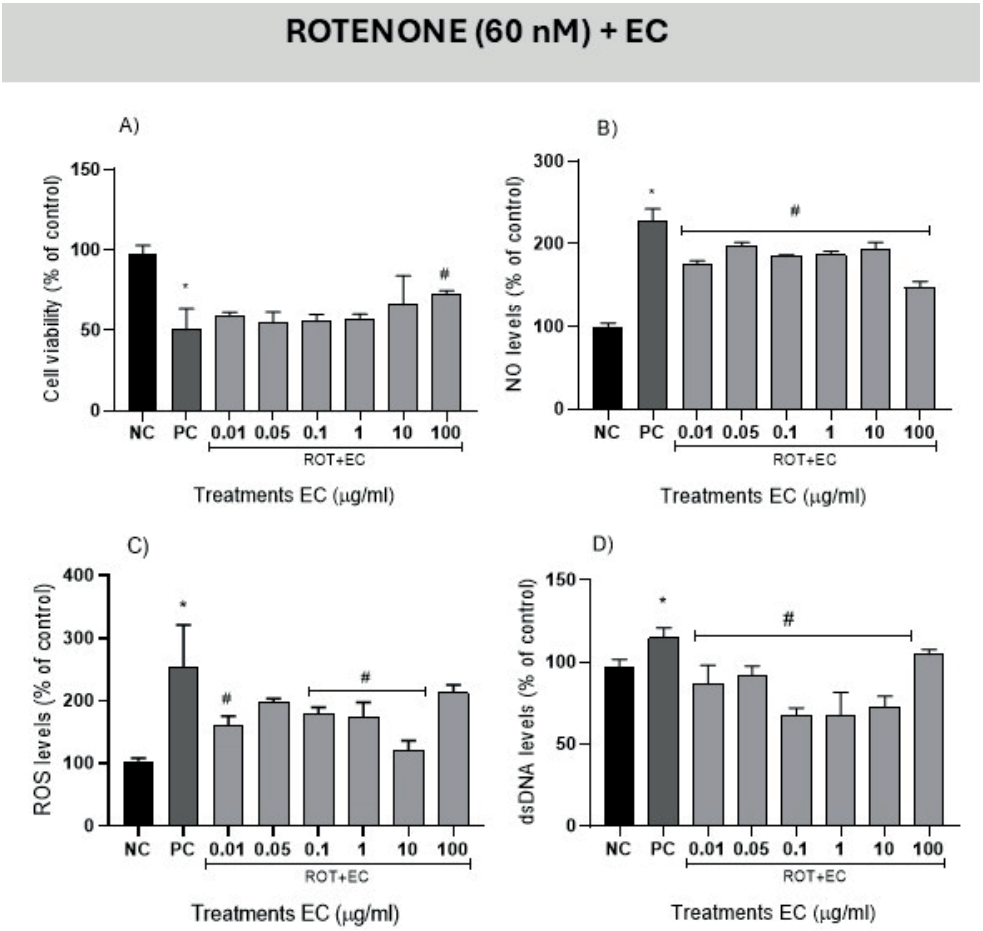


Concentration curve of mitochondrial damage-inducing agents: rotenone, sodium azide, and sodium nitroprusside, respectively. (A and E) Cell viability analysis (24 h); (I) Cell proliferation analysis (48 h); (B, F, and J) Measurement of nitric oxide release levels; (C, G, and K) Measurement of released ROS; (D, H, and L) Quantification extracellular dsDNA. CN: Negative control (cells under conventional culture conditions). Results are expressed as percentages relative to the negative control (100%), and statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *indicates comparison with the negative control, with $p < 0.05$.

In this section, the results related to the effects of epicatechin (EC) are presented, evaluating the same parameters mentioned in the previous models. An initial study previously conducted by the group assessed the safety profile of EC in VERO cells, a cell line derived from the African green monkey kidney, and demonstrated that the molecule has a satisfactory safety profile for use, without increasing cellular damage parameters and showing no cytotoxicity (Davidson *et al*., 2024).

Subsequently, monocytes were exposed to damage induced by rotenone (60 nM) for 24 hours, which, as expected, reduced cell viability. Regarding treatments with EC, only the concentration of 100 μg/mL was able to significantly mitigate cell death. When analyzing nitric oxide (NO) release, EC was highly effective, reducing the amount of NO released at all tested concentrations. Furthermore, the results also indicate that EC significantly decreased levels of reactive oxygen species (ROS) and extracellular dsDNA compared to the positive control (PC, consisting of the stressor agent alone). These results are shown in Figure 2.

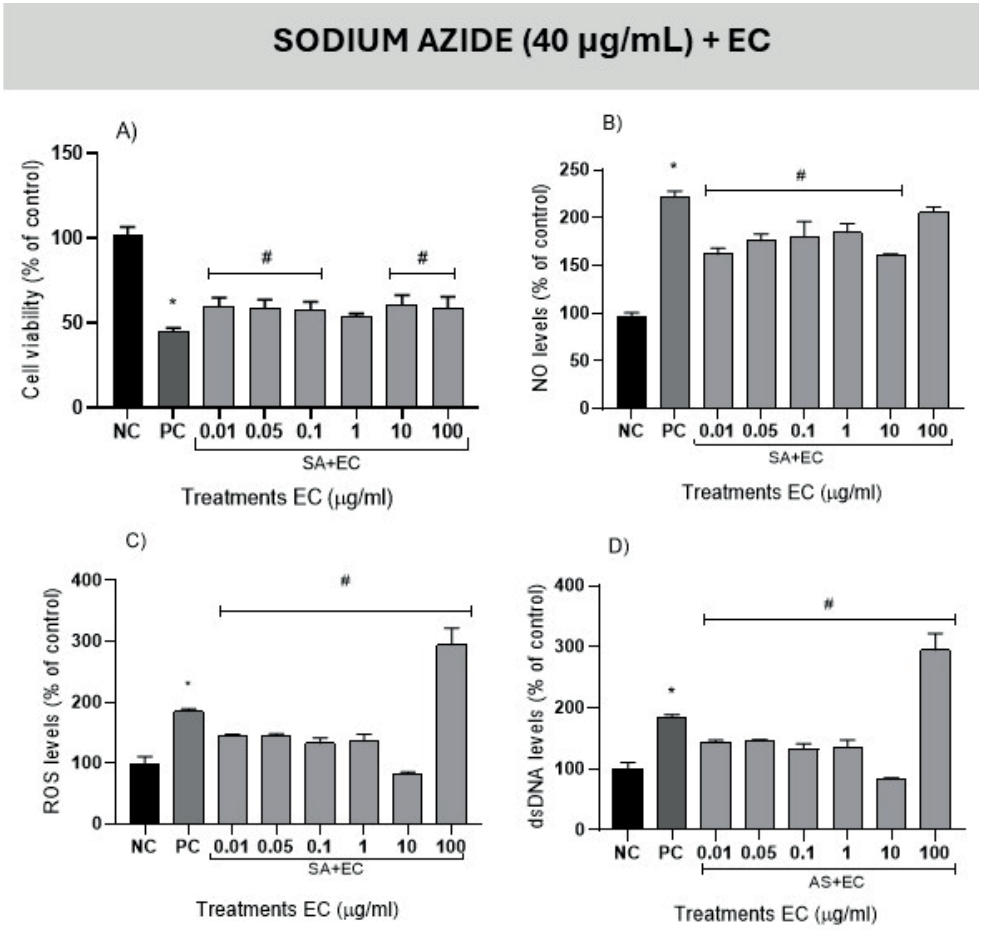
Figure 2 - Concentration curve of EC for reversal of damage induced by 60 nM rotenone in THP-1 cells.



THP-1 cells were exposed to rotenone followed by treatment with different concentrations of EC. (A) Cell proliferation level; (B) Measurement of nitric oxide levels; (C) Measurement of ROS; (D) Quantification of extracellular dsDNA. CN: Negative control (cells under conventional culture conditions). CP: Rotenone control (60 nM). Results are expressed as percentages relative to the negative control (100%), and statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. *indicates comparison with CN, where $p < 0.05$. # indicates comparison with CP, where $\#p < 0.001$.

Furthermore, when exposed to sodium azide (40 μg/mL) for 24 hours, the monocytes exhibited reduced cell viability and increased levels of nitric oxide (NO), reactive oxygen species (ROS), and extracellular dsDNA release compared to the negative control (NC). On the other hand, as shown in Figure 3, the results indicate that most concentrations of EC were effective in reversing these conditions.

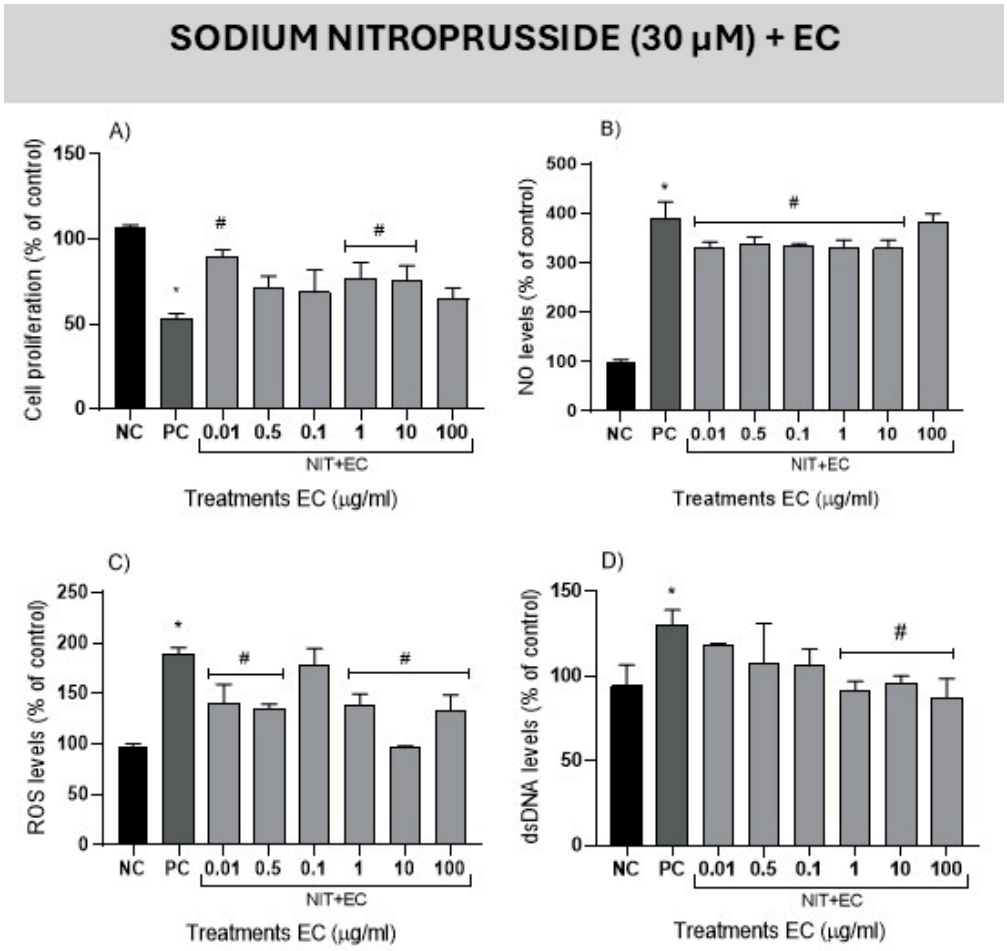
Figure 3 - EC concentration curve for reversal of sodium azide-induced damage in THP-1 cells.



THP-1 cells were exposed to sodium azide (40 µg/mL) followed by addition of different concentrations of EC. (A) Cell proliferation level; (B) Measurement of NO levels; (C) Measurement of ROS levels; (D) Quantification of extracellular dsDNA. NC: Negative control (cells under standard culture conditions). PC: Sodium azide control (40 µg/mL). Results are expressed as a percentage relative to the negative control (100%), and statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. *indicates comparison with NC, where $p < 0.05$. # indicates comparison with SAC, where $\#p < 0.001$.

Additionally, cells were subjected to damage induced by sodium nitroprusside. The results demonstrate that EC at concentrations of 0.01, 1, and 10 μg/mL was able to protect the cells from induced cell death, as well as reduce levels of nitric oxide (NO) and reactive oxygen species (ROS) release. Regarding extracellular dsDNA measurement, concentrations ranging from 1 to 100 μg/mL showed efficacy, as evidenced in Figure 4.

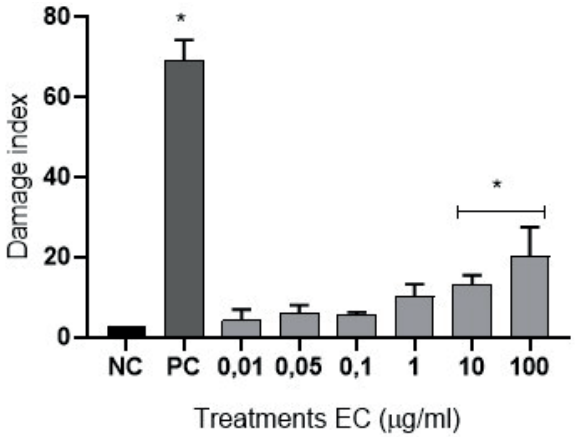
Figure 4 - EC concentration curve for reversal of sodium nitroprusside-induced damage in THP-1 cells.



THP-1 cells were exposed to sodium nitroprusside (30 µM) followed by addition of different concentrations of EC. (A) Cell proliferation level; (B) Measurement of NO levels; (C) Measurement of ROS levels; (D) Quantification of extracellular dsDNA. NC: Negative control (cells under standard culture conditions). PC: Sodium nitroprusside control (30 µM). Results are expressed as a percentage relative to the negative control (100%), and statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. * indicates comparison with NC, where $p < 0.05$. # indicates comparison with SNC, where $\#p < 0.001$.

Currently, many studies focus on investigating the safety and genotoxicity of natural compounds and isolated bioactive molecules. In this context, the alkaline comet assay serves as a complementary method to the previous results. It was observed that epicatechin (EC) is a DNA-safe compound, as it did not cause single-strand DNA damage, showing significant results only at concentrations of 10 and 100 μg/mL compared to the negative control (NC). The positive control used for assay validation was hydrogen peroxide (H_2O_2) at a concentration of 3 μM, where a higher percentage of damaged DNA was observed compared to the NC ($p < 0.05$).

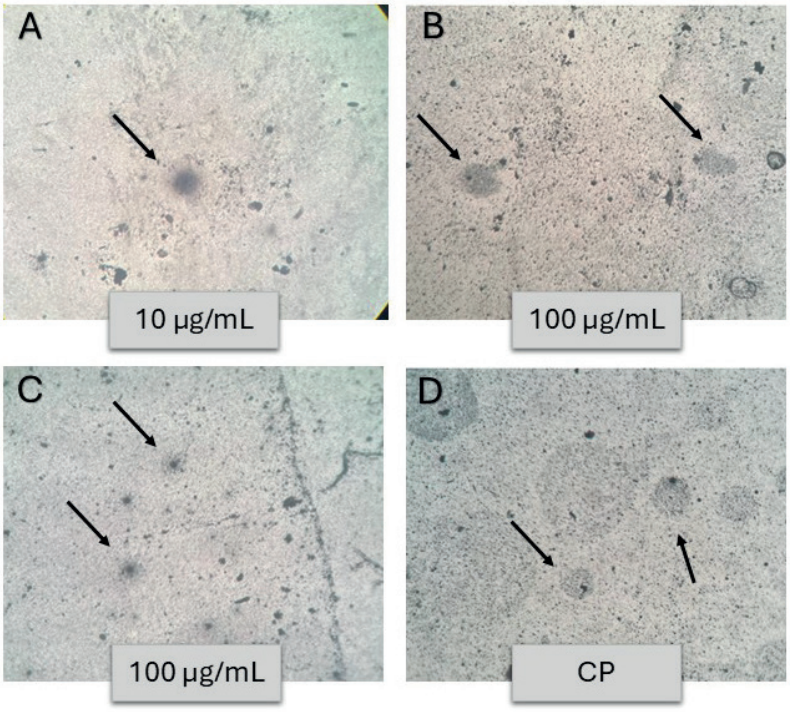
Figure 5 - DNA damage index in alkaline comet assay.



DNA damage index (%) in cells treated with different concentrations of EC. NC: Negative control (cells under standard culture conditions, RPMI 1640 medium). PC: Positive damage control (H2O2 3 µM). Results are expressed as DNA damage index, and statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. * indicates comparison with the negative control, where $p < 0.05$.

The study by Haza and Morales (2011) reports results in which epicatechin (EC) acted as a protective agent by reducing damage induced by heterocyclic amines, protecting DNA strands in HepG2 cell lines as evaluated by the alkaline comet assay. These findings indicate that EC, in addition to not causing significant DNA damage at concentrations below 1 ***µ***g/mL, may also serve as a cytoprotective agent. Figure 6 shows the levels of damage observed microscopically.

Figure 6 - Examples of alkaline comet assay tails.



Examples of alkaline comet assay tails. Single-strand DNA damage index in THP-1 cells treated with a concentration curve of EC. Images show concentrations of 10 and 100 µg/mL as well as the PC. Analysis was performed using an optical microscope with a 40x objective. Comets are indicated by arrows. (A) Tail of a comet from cells treated with EC 10 µg/mL; (B and C) Different representative fields of the same condition (EC 100 µg/mL); (D) Representation of the PC (H2O2 3 µM), where apoptotic areas can be observed.

Overall, the results demonstrate that exposure of THP-1 cells to rotenone, sodium azide, and sodium nitroprusside induced changes in parameters associated with cellular damage in a concentration- and agent-dependent manner. The concentrations of 60 nM rotenone, 40 μg/mL sodium azide, and 30 μM sodium nitroprusside were selected for subsequent assays based on their ability to induce demonstrative oxidative damage effects without causing complete cell death. On the other hand, the results indicate that epicatechin (EC) was effective in protecting the cells against induced oxidative damage, acting to preserve cell viability and reduce levels of nitric oxide (NO), reactive oxygen species (ROS), and extracellular dsDNA. Additionally, EC was characterized as a DNA-safe compound based on the alkaline comet assay analysis.

CONCLUSION

The relevance of mitochondrial modulation in cellular homeostasis and its importance in many neurodegenerative, metabolic, neuropsychiatric diseases, and neoplasms is well established in numerous scientific studies. On the other hand, various investigations have demonstrated the health benefits of natural products, highlighting them in research due to their biological potential in diverse conditions. Based on the results obtained, it can be stated that artificial alterations in monocyte cellular homeostasis were successfully induced by exposure to different stressor agents, with effective concentrations and incubation periods identified for inducing oxidative imbalance in exposed cells. Furthermore, epicatechin (EC) proved to be a potential therapeutic agent, as this bioactive compound was able to reverse the cellular alterations induced by stressor agents at most concentrations evaluated, suggesting a possible role in the recovery of compromised mitochondrial function. Finally, it can also be said that EC exhibited a satisfactory in vitro safety profile, as it did not cause cellular or DNA damage.

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