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Effect of jaboticaba and pequi extracts on gene expression of antioxidant enzymes in C2C12 mouse muscle cells

Efeito dos extratos de jabuticaba e pequi na expressão gênica de enzimas antioxidantes em células musculares C2C12 de camundongos

Efecto de los extractos de jaboticaba y pequi sobre la expresión génica de enzimas antioxidantes en células musculares de ratón C2C12

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Abstract
Jaboticaba and pequi fruits contain bioactive compounds, such as polyphenols, which present antioxidant actions; however, the molecular mechanisms by which these effects are achieved are not fully elucidated. In the present study, mouse muscle cells (C2C12), induced or not by oxidative stress with hydrogen peroxide (H2O2), were treated with jaboticaba peel extract (JPE) and pequi aqueous extract (PAE) in order to evaluate their influence on expression of the following antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione s-transferase (GST) and glutathione peroxidase 1 (GPX1). Treatments with JPE (150 µg gallic acid equivalents mL-1 extract) and PAE (30 µg GAE mL-1 extract) increased CAT and GST mRNAs expression in muscle cells. In cells induced by oxidative stress with addition of H2O2, peroxide detoxification action attributed to the GST enzyme was verified. The extracts beneficial effects may be due to the synergistic activities of their various phenolic compounds and other constituents.
Keywords: Antioxidant action; Messenger RNA; Phenolic compounds; Plant extracts; Oxidative stress; Cytotoxicity.

Resumo
Frutos de jabuticaba e pequi contêm compostos bioativos, como polifenóis, que apresentam ação antioxidante; entretanto, os mecanismos moleculares pelos quais esses efeitos são alcançados não estão totalmente elucidados. No presente estudo, células musculares de camundongo (C2C12), induzidas ou não por estresse oxidativo com peróxido

de hidrogênio (H2O2), foram tratadas com extrato de casca de jabuticaba (JPE) e extrato aquoso de pequi (PAE) para avaliar sua influência na expressão das seguintes enzimas antioxidantes: catalase (CAT), superóxido dismutase (SOD), glutationa s-transferase (GST) e glutationa peroxidase 1 (GPX1). Os tratamentos com JPE (150 µg equivalentes de ácido gálico mL-1 extrato) e PAE (30 µg GAE mL-1 extrato) aumentaram a expressão de mRNAs de CAT e GST em células musculares. Em células induzidas por estresse oxidativo com adição de H2O2, verificou-se a ação de desintoxicação do peróxido atribuída à enzima GST. Os efeitos benéficos dos extratos podem ser devido às atividades sinérgicas de seus diversos compostos fenólicos e outros constituintes.
Palavras-chave: Ação antioxidante; RNA mensageiro; Compostos fenólicos; Extratos vegetais; Estresse oxidativo; Citotoxicidade.

Resumen

Los frutos de jaboticaba y pequi contienen compuestos bioactivos, como polifenoles, que presentan acciones antioxidantes; sin embargo, los mecanismos moleculares mediante los cuales se logran estos efectos no están completamente aclarados. En el presente estudio, células de músculo de ratón (C2C12), inducidas o no por estrés oxidativo con peróxido de hidrógeno (H2O2), fueron tratadas con extracto de piel de jaboticaba (JPE) y extracto acuoso de pequi (PAE) para evaluar su influencia en la expresión de las siguientes enzimas antioxidantes: catalasa (CAT), superóxido dismutasa (SOD), glutatión s-transferasa (GST) y glutatión peroxidasa 1 (GPX1). Los tratamientos con JPE (150 µg equivalente de ácido gálico mL-1 extracto) y PAE (30 µg GAE mL-1 extracto) aumentaron la expresión de ARNm de CAT y GST en las células musculares. En células inducidas por estrés oxidativo con adición de H2O2, se verificó la acción de desintoxicación del peróxido atribuida a la enzima GST. Los efectos beneficiosos de los extractos pueden deberse a las actividades sinérgicas de sus diversos compuestos fenólicos y otros constituyentes.
Palabras clave: Acción antioxidante; ARN mensajero; Compuestos fenólicos; Extractos de plantas; Estrés oxidativo; Citotoxicidad.

1. Introduction

Oxidative stress is a phenomenon triggered by the imbalance between the production and elimination of reactive oxygen species (ROS) in cells and tissues, which results in ROS accumulation due to the inability of the biological system to detoxify such reactive products (Pisoschi et al., 2021). Reactive oxygen species play several physiological roles, including cell signaling. However, ROS excess is responsible for several harmful actions on the body, such as increased membrane lipid peroxidation levels (Bahja & Dymond, 2021), increased protein carbonylation, and DNA damage (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006) which ultimately alter and impair intracellular metabolism and may even cause cell death (Dionísio, Amaral, & Rodrigues, 2021). Therefore, factors including oxidative stress, enzymes or biomarkers responsible for intracellular homeostatic balance are of great interest.

Plants produce several secondary metabolites, including polyphenols, which act as antioxidant source and protect them from the deleterious effects of oxidative stress (Scartezzini & Speroni, 2000). Over the past 10 years, consumer interest has increased regarding a range of high-polyphenol foods (Panza et al., 2016). These compounds comprise a heterogeneous group of molecules that differ according to their chemical structure (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004), and may be more efficient than other antioxidants (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005).

Brazilian jaboticaba fruit (Myrciaria jaboticaba (Vell.) Berg.) is one of the most studied and promising species of Myrciaria genus, being native and widespread in Brazil, but also produced in Bolivia, El Salvador, Honduras and Paraguay (Borges, Cardoso, & Silveira, 2014). Jaboticaba peel extract has been studied and its antioxidant function is mediated by a wide range of compounds with phenolic functional properties (Neri-Numa, Sancho, Pereira, & Pastore, 2018; Plaza et al., 2016; Tarone, Goupy, Ginies, Marostica Junior, & Dufour, 2021).

Pequi (Caryocar brasiliense Camb.) is another Brazilian fruit that has aroused the interest of researchers, due to the presence of bioactive compounds in its composition. This is a native fruit from Brazilian Cerrado, being an important income and food sources for the population from this region (Leão, Franca, Oliveira, Bastos, & Coimbra, 2017). Studies have reported the presence of phenolic compounds in pequi aqueous extract (Machado, Mello, & Hubinger, 2013; Roesler, Catharino, Malta, Eberlin, & Pastore, 2008) and its high antioxidant capacity (Machado, Mello, & Hubinger, 2015).

Jaboticaba peel has been used as a therapy for several diseases, such as obesity (Lenquiste et al., 2015), cardiovascular disease (Sá et al., 2014) and liver steatosis (Batista et al., 2018) and has also shown antiproliferative effects against leukemia (K-562) and prostate cancer (PC-3) (Leite-Legatti et al., 2012). Regarding Pequi fruit, it has be shown that it may decrease hydroxyl radical formation, present anticlastogenic activity, and inhibit bleomycin-induced DNA damage in mice (Khouri et al., 2007). Although the underlying mechanisms relevant to these diseases' pathogenesis have not yet been fully elucidated, ROS may significantly contribute, and the therapeutic functions of jaboticaba peel and pequi pulp are closely linked to their antioxidant efficiency (Batista et al., 2014; Leite-Legatti et al., 2012; Lenquiste et al., 2015).

Studies have reported that skeletal muscle cells often become ROS targets under several conditions (Khan et al., 2008; Wataru & Kunihiro, 2011), leading, to some extent, to cellular dysfunction (Ohta et al., 2011; Yokota et al., 2009). Acute muscle injury in rats has also been found to result in skeletal muscle fiber disruption, oxidative stress, and inflammation (Myburgh, Kruger, & Smith, 2012). Exposure of C2C12 myotubes to ROS, such as hydrogen peroxide (H2O2), results in muscle protein loss and fiber atrophy (Gilliam et al., 2012; McClung, Judge, Talbert, & Powers, 2009). Recent studies have shown that extracts from different antioxidant fruits are a viable way to relieve ROS negative effects on skeletal muscle (Chang et al., 2017; Kerasioti, Stagos, Tzimi, & Kouretas, 2016; Priftis et al., 2018).

In this context, it is relevant to perform studies aiming to identify extracts and compounds that may contribute to reverse or slow down cellular damage. Increasing research on the role of these compounds in gene expression regulation becomes crucial for the development of new nutritional therapies. In addition, it is of great importance to elucidate the intracellular mechanisms by which these compounds exert their therapeutic effects, either in preventive or therapeutic forms, attempting to reestablish the redox balance in the organism. In this sense, aimed to determine the ability of jaboticaba peel extract (JPE) and pequi aqueous extract (PAE) in the reduction of oxidative stress in skeletal muscle cells that often become ROS targets under different conditions.

2. Methodology

This study is an applied research with a quantitative approach, with experimental technical procedures (Gil, 2008). Quantitative research uses structured procedures and formal instruments to collect data under controlled conditions. In addition, it emphasizes objectivity in data collection and analysis, which is performed through statistical procedures (Gerhardt & Silveira, 2009; Pereira, Shitsuka, Parreira, & Shitsuka, 2018). Statistical method is based on the application of the statistical theory of probability. Thus, it is possible to determine, in numerical terms, the probability of correctness of a given result, as well as the margin of error of a value obtained, contributing significantly to the conclusions obtained (Gil, 2008).

2.1 Reagents and solutions

All reagents used in this study were classified as of analytical purity grade: citric acid, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 1% agarose gel, ethanol and methanol, purchased from Vetec (Duque de Caxias, RJ, Brazil); sodium carbonate; Folin-Ciocalteau reagent, purchased from Êxodo Científica (Hortolândia, SP, Brazil); 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate, gallic acid, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (trolox), gentamicin sulfate and fetal bovine serum (FBS), purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA); Dulbecco's Modified Eagle's medium (DMEM), sodium bicarbonate, penicillin and streptomycin, purchased from Gibco (Grand Island, NY); and [3- (4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazolium] bromide (MTT) purchased from Invitrogen, Thermo Fisher Scientific (Oregon, USA). The aqueous solutions were prepared with ultrapure water (18.2 MΩ·cm resistivity at 25 °C), obtained by the Milli-Q deionization system (Millipore, Bedford, MA, USA).

2.2 Extracts preparation

Jaboticaba fruits (*Myrciaria jaboticaba* Vell) Berg.) came from the Fruit Production Sector of Universidade Federal de Viçosa, Viçosa, MG, Brazil. Jaboticaba peels were obtained from the processing of *in natura* fruits, which were manually pulped, and their peels frozen in liquid nitrogen and then kept frozen (-18 ºC to -20 ºC). For extract preparation, about 5 g of jaboticaba peel were triturated in a domestic blender with 70% (v/v) ethanol (1:2 peel:solvent) and acidified with 6% citric acid solution until reaching pH 3.0. Then, the mixture was left to stand protected from light and refrigerated (4 °C ± 1 °C) for 24 hours. The material was filtered on filter paper. The resulting extract was concentrated until total elimination of solvent in rotary evaporator (Laborota, 4000) at 40 °C, protected from light, until reaching a total soluble solids content of approximately 9 ºBrix (Silva, Stringheta, Teofílo, & Oliveira, 2013).

Pequi pulp (*Caryocar brasiliense* Camb.) was obtained from the processing of *in natura* ripe fruits, which came from Santana de Pirapama municipality, MG, Brazil. A 0.25 df bonina pulping machine (Itametal/NPC Equipamentos, Itabuna, BA, Brazil) was used to obtain the pulp. The pequi aqueous extract was prepared from the pulp, following Machado et al. (2013) recommendations, with some modifications. In this way, 20 g of pequi pulp, previously thawed under refrigeration, were weighed and distilled water was added at a ratio of 1:3 (m/m, pulp:solvent). The mixture was homogenized in an amber flask using a magnetic stirrer (DT3110H, DiagTech®, São Paulo, SP, Brazil) at 750 rpm for 2 hours at room temperature. Then, the material was centrifuged at 15,000 rpm for 15 minutes at 25 °C in a centrifuge (Beckman Coulter J2-MC, Inc., Fullerton, CA, USA) and the supernatant was vacuum filtered on filter paper.

2.3 Cell culture

C2C12 myoblasts (CRL - 1772, adult mouse satellite cells) were obtained from the American Type Culture Collection (ATCC, Rockville, USA) and grown in DMEM medium supplemented with 10% FBS, sodium bicarbonate (0.0025 g mL^{-1}), gentamicin sulfate (10 g mL^{-1}), penicillin (100 U mL^{-1}) and streptomycin (100 g mL^{-1}) in a modified atmosphere of 95% air and 5% CO_2 at 37 °C. The pH of the cell culture medium was adjusted to 7.08, according to the manufacturer's recommendations.

In order to use the cells in the assays, trypsinization was performed through washing with addition of 5 mL trypsin-EDTA for 5 minutes at 37 °C and slight shaking to detach the cells. Subsequently, cells were resuspended in 10 mL of supplemented DMEM medium and centrifuged at 400 x g (Megafuge 40R, Thermo Scientific). At the end, the supernatant was discarded and the cell pellet was resuspended in supplemented DMEM medium for cells counting (Countess Automated Cell Counter, Invitrogen), in order to carry out cells plating in 6- and 96-well culture plates.

2.4 Extracts cytotoxicity

Prior to the determination of plant extracts cytotoxicity, the phenolic compounds content was determined and the antioxidant capacity was evaluated (ABTS and DPPH assays), according to the methodology described by Re et al. (1999) and Brand-Williams, Cuvelier and Berset (1995), respectively, with modifications described by Pinheiro et al. (2018). Results were expressed as trolox equivalent (µmol trolox mL^{-1} extract).

The total phenolic compounds content was also evaluated based on Swain e Hills (1959) assay, and from a standard gallic acid curve, the results were expressed as gallic acid equivalents (µg GAE mL^{-1} extract).

To investigate the extracts cytotoxicity, 3 x 10^4 C2C12 cells were cultured per well until reaching 70% confluence in 96-well microplates, in DMEM medium, and exposed to plant extracts containing different total phenolic concentrations (JPE: 0, 10, 70, 90, 120, 150 µg GAE mL^{-1} extract, and PAE: 0, 10, 30, 50 µg GAE mL^{-1} extract) for 24 hours. Extract-free DMEM medium was used as a control. After this period, each cell culture microplate well containing the treatments was washed with

100 µL PBS and then 10 µL MTT at 5 mg mL^{-1} dissolved in PBS were added. The MTT stock solution was stored under refrigeration and protected from light. Subsequently, 10 µL MTT were added to the culture microplate wells and incubated at 37 °C for 4 to 6 hours until the purple formazan crystals became visible (Bahuguna, Khan, Bajpai, & Kang, 2017; Mosmann, 1983; Van Meerloo, Kaspers, & Cloos, 2011).

Subsequently, the cell growth medium with MTT was removed and 100 µL DMSO was added for crystal solubilization, shaking the microplate for 5 minutes at 900 rpm on a shaker (High-Speed microplate shaker, Illumina, San Diego, California, USA). The culture plate was kept at room temperature in absence of light for 5 minutes for color stabilization. Reading was performed on a spectrophotometer (Multiskan Spectrum, Thermo Scientific, Oregon, USA) at 540-570 nm (ATCC, 2011; Mosmann, 1983).

2.5 Stress assay and cell recovery

C2C12 cells were cultured as previously described and plated in 6-well plates upon reaching 70% confluence. Each well had the following treatments: JPE (total phenolic concentration of 150 µg GAE mL^{-1} extract), PAE (total phenolic concentration of 30 µg GAE mL^{-1} extract) and control (supplemented DMEM medium). To simulate oxidative stress, cells were pretreated with 200 mmol L^{-1} H_2O_2 for 15 minutes, washed with PBS and then treated with JPE and PAE as previously described. After these processes, the medium in which the cells were cultured was removed, the cells were washed with PBS and RNA was extracted.

2.6 RNA extraction and cDNA synthesis

Cultures total RNA extraction was performed with Trizol® (InvitrogenTM, Thermo Fisher Scientific, Oregon, USA), according to manufacturer's protocol. Total RNA content was estimated using spectrophotometer (NanoVue GE Healthcare Life Sciences Inc., Little Chalfont, United Kingdom) and RNA integrity was determined on 1% agarose gel.

The cDNAs used in real-time polymerase chain reaction (PCR) analyses were synthesized by reverse transcription reaction using the commercial reagent set GoScriptTM Reverse Transcription System (Promega Corporation, Madison, WI, USA), following manufacturer's recommendations. Their concentration was determined by spectrophotometer (NanoVue GE Healthcare Life Sciences Inc., Little Chalfont, United Kingdom). One µg RNA was used for reverse transcription reaction using Promega's GoScript Reverse trascriptase kit according to manufacturer's recommendations (Promega Corporation, Madison, USA). Afterwards, the samples were stored at -20 °C until qPCR was performed.

The β-actin, GAPDH, CAT, GST, GPX1 and SOD primers were designed in PrimerQuest tool (Integrated DNA Technologies, Inc., Coralville, IA, USA) through sequences obtained from GeneBank database, as presented in Table 1.

Table 1. Gene names, primer sequences, and primer pair accession codes of each target gene.

Gene	Sequence	NCBI accession number
Beta actin (BACT)	F:CTAGACTTCGAGCAGGAGAT R: CCGCTCGTTGCCAATAG	NM_007393.5
Catalase (CAT)	F: GGATTCCTGAGAGAGTGGT R: CCTTTGCCTTGGAGTATCTG	NM_009804.2
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: GGGTGCAGCGAACTTTAT R: CCCTCACAATTTCCATCCC	NM_001289726.1
Glutathione S-Transferase (GST)	F: CCCTGGTTGAACTCCTCTA R: GTTGCTGACTCTGCTTCTC	NM_019946.5
Glutathione Peroxidase (GPX1)	F: GGGACTACACCGAGATGAA R: CTGGTGTCCGAACTGATTG	NM_008160.6
Superoxide Dismutase (SOD)	F:CTCAAGGGTATGGGCAGTAG R:GAGTTCAGAGGATAGCTGTAGG	NM_011434.2

Source: Authors.

β-actin and GAPDH genes were used in normalization analyses to minimize possible variations regarding the amount of initial mRNA and the reverse transcription efficiency. Endogenous gene selection was based on the amplification efficiency of the candidate genes, through the calculation of efficiency for each primer pair (at 100, 200 and 400 mmol L^{-1} concentrations), according to Eq. (1):

$$E = 10^{\frac{1}{A}} - 1 \quad (1)$$

where E is the reaction efficiency, which ranged from 0.80 to 0.97 for the endogenous genes tested; and A is the slope of the line.

In addition, the amplification and dissociation curves profiles and genes amplification stabilities between treatments were verified using Genorm and Normfinder algorithms. β-actin was chosen as the best endogenous gene due to its greater stability between treatments.

Real-time polymerase chain reactions (RT-qPCR) were performed in duplicate using the ABI Prism 7300 Sequence Detection Systems thermocycler (Applied Biosystems, Foster City, CA, USA), through Relative Quantification method using as detection the SYBR® Green system (Applied Biosystems - Foster City, CA, USA) and the GoTaq® qPCR Master Mix Kit (Promega corporation, Madison, USA). The PCR reactions were submitted to the cycles protocol according to the following program: 95 ºC for 3 minutes, 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. The resulting threshold cycle (Ct) values were posteriorly normalized by the delta-ct (ΔCt) method, based on Ct values obtained for the endogenous control gene (β-actin). Relative levels of gene expression were quantified by the DDCt method, which provides a ratio of target gene expression to uniformly expressed endogenous gene levels (Livak & Schmittgen, 2001).

2.7 Statistical analysis

The assays were performed in triplicate and values expressed as mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by Sidak test for cell viability or Newman-Keuls test for gene expression, using GraphPad Prism statistical software (version 6.02; GraphPad Software, San Diego, CA, USA). Significant differences were indicated as $p < 0.05$.

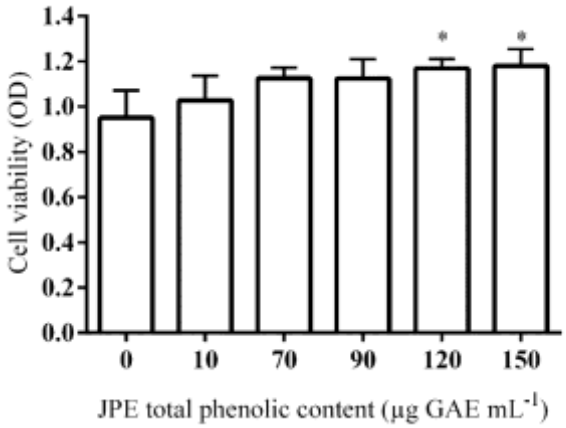
3. Results and Discussion

3.1 Extracts cytotoxicity

The JPE and PAE extracts, used for analyses in C2C12 cells, presented total phenolic content of 4,935.00 ± 1.34 and 266.75 ± 0.74 µg GAE mL^{-1} extract, respectively. For the antioxidant capacity by ABTS assay, 89.31 ± 0.53 and 1.21 ± 0.17 µmol trolox mL^{-1} extract were obtained for JPE and PAE, respectively. Regarding the DPPH assay, 82.04 ± 0.34 and 1.19 ± 0.07 µmol trolox mL^{-1} extract were obtained for JPE and PAE, respectively. The interest in South American native plant species has grown in recent years due to their health benefits (Oliveira, Yamada, Fagg, & Brandão, 2012). Currently, greater attention has been given to the antioxidant capacity of natural products, and extracts from jaboticaba peel (Batista et al., 2018; Calloni, Dall, Soares, Siqueira, & Moura, 2018) and pequi (Khouri et al., 2007; Leão et al., 2017; Machado et al., 2013) have emerged as antioxidant sources.

From the previously described data, it was possible to investigate JPE-induced cytotoxicity (Figure 1).

Figure 1. Cell viability (C2C12 cells) in response to the addition of jaboticaba peel extract (JPE) at different total phenolic concentrations.

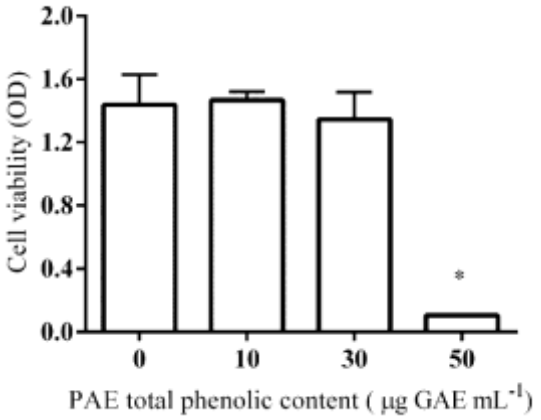


Data are presented as mean ± standard deviation of three independent experiments. * $p < 0.05$ versus control, by Sidak test. GAE = gallic acid equivalent. OD = optical density. Source: Authors.

Figure 1 shows C2C12 cells viability in response to the addition of JPE at different total phenolic concentrations. C2C12 cells were incubated with different phenolic concentrations in the extract (10, 70, 90, 120 and 150 µg GAE mL^{-1} extract) and cell viability was determined by MTT assay. After 24 hours of incubation, concentrations of 10, 70 and 90 µg GAE mL^{-1} extract did not show any significant cytotoxic effect on C2C12 cells compared to control ($p > 0.05$). However, at 120 and 150 µg GAE mL^{-1} extract concentrations, there was a significant increase in cell viability compared to control ($p < 0.05$) (Figure 1).

To evaluate PAE effect on C2C12 cells survival, the cells were incubated with different phenolic concentrations in the extract (10, 30 and 50 µg GAE mL^{-1} extract) (Figure 2).

Figure 2. Cell viability (C2C12 cells) in response to the addition of pequi aqueous extract (PAE) at different total phenolic concentrations.



Data are presented as mean ± standard deviation of three independent experiments. * p <0.05 versus control, by Sidak test. GAE = gallic acid equivalent. OD = optical density. Source: Authors.

Figure 2 shows C2C12 cells viability in response to the addition of PAE at different total phenolic concentrations. After 24 hours of incubation, concentrations of 10 and 30 µg GAE mL^{-1} extract did not show any significant cytotoxic effect on C2C12 cells ($p > 0.05$). However, the concentration of 50 µg GAE mL^{-1} extract significantly reduced C2C12 cells viability ($p < 0.05$) (Figure 2). In this way, total phenolic concentrations selected for subsequent analyses were 150 µg GAE mL^{-1} JPE and 30 µg GAE mL^{-1} PAE.

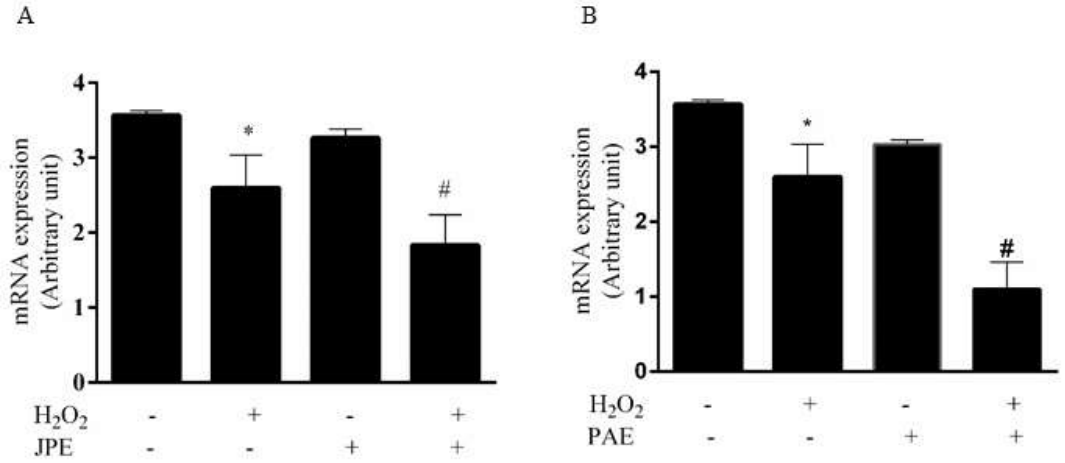
3.2 Gene expression analysis

Expression of genes encoding antioxidant enzymes is crucial to maintain cellular homeostasis (Zhang et al., 2016). In this sense, this is the first study exploring the effect of the addition of jaboticaba peel and pequi pulp aqueous extracts on messenger RNA (mRNA) expression of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX1) and glutathione s-transferase (GST) in a mouse muscle cell line (C2C12).

The SOD enzyme is the first defense line against oxygen-derived free radicals, catalyzing the superoxide anion ($O2^{\bullet-}$) dismutation into H_2O_2, which is latter transformed into H_2O and O_2 by CAT enzyme (Kaynar et al., 2005).

Figure 3 shows JPE and PAE effects on superoxide dismutase mRNA expression in C2C12 muscle cells without stress (control) and with hydrogen peroxide stress induction. After stress induction in cells with 200 mmol L^{-1} H_2O_2, SOD mRNA expression decreased compared to control cells ($p < 0.05$). Decrease in gene expression ($p < 0.05$) was also observed in stressed and JPE-treated cells compared to stressed cells without JPE addition.

Figure 3. Extracts effect on superoxide dismutase (SOD) mRNA expression in C2C12 muscle cells without stress (control) and with hydrogen peroxide (H_2O_2) stress induction.



A) Effect of jaboticaba peel extract (JPE); B) Effect of pequi pulp aqueous extract (PAE). Total RNA was extracted and mRNA levels were assessed by real time polymerase chain reaction (RT-PCR). Data are expressed as mean ± standard deviation. * $p < 0.05$ versus control, # $p < 0.05$ versus H_2O_2 stressed cells, by Newman-Keuls test. Source: Authors.

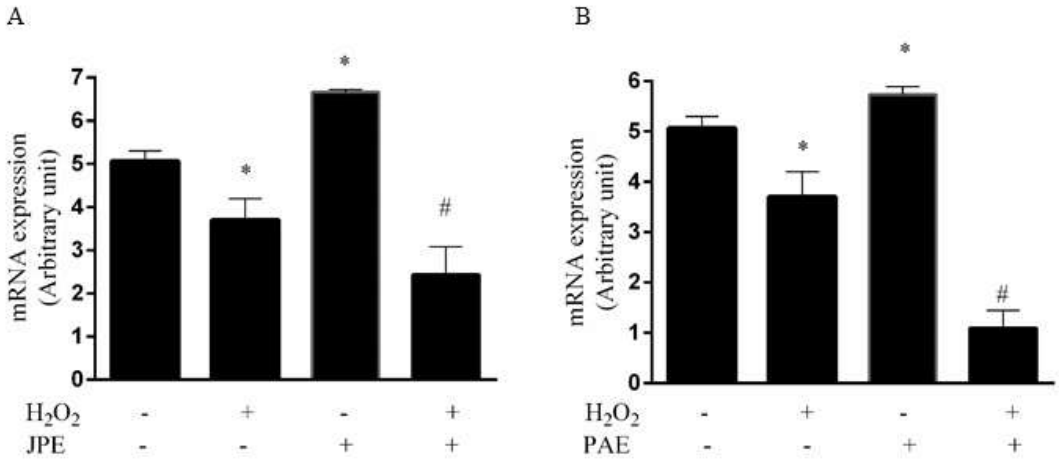
In Figure 3A, there was no effect ($p > 0.05$) of JPE addition on SOD gene expression in non-stressed cells compared to the control. In Figure 3B, the PAE-treated C2C12 cells showed statistically similar behavior to JPE for SOD mRNA expression. In accordance with the results of the present study, other studies have reported that phenolic compounds from grape extracts, for example, did not affect SOD mRNA expression (Fernández-Iglesias et al., 2014), as well as 17 µg mL^{-1} polyphenols from coffee extract did not affect SOD gene expression in C2C12 cells (Priftis et al., 2018).

The hypotheses is that SOD mRNA expression decreases when cells are stress-induced because H_2O_2 is the final product of SOD action and acts as its expression inhibitor. Due to the high concentrations of the final product, the cell associates that the enzyme concentrations are high, and SOD expression is consequently reduced (Mayer & Falkinham, 1986; Xianyong, Dun, & Weidong, 2017).

CAT is an antioxidant enzyme that catalyzes the conversion of H_2O_2, a reactive molecule that leads to the production of the hydroxyl radical (OH·), H_2O and O_2 (Xianyong et al., 2017).

Figure 4 shows JPE and PAE extracts effects on catalase mRNA expression in C2C12 muscle cells.

Figure 4. Extracts effect on catalase (CAT) mRNA expression in C2C12 muscle cells without stress (control) and with hydrogen peroxide (H_2O_2) stress induction.



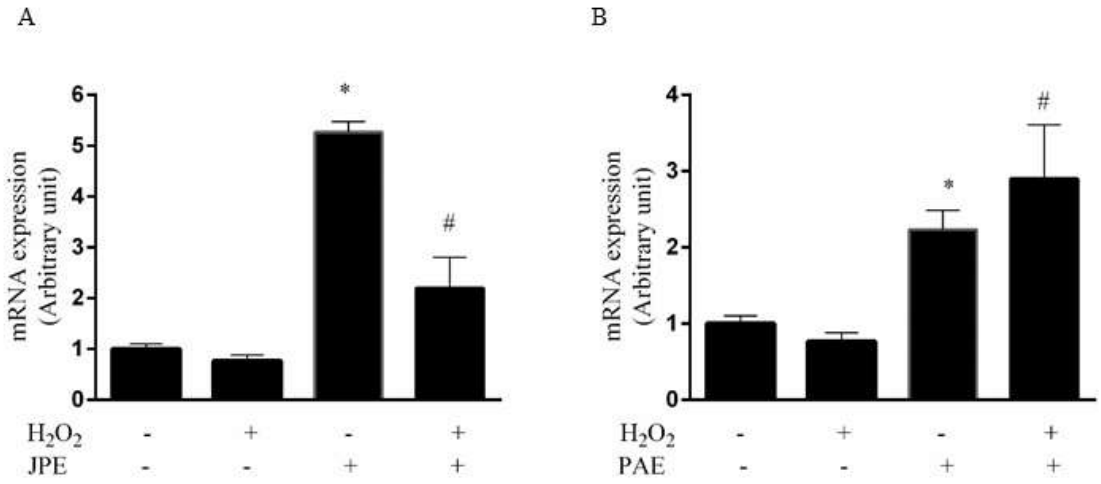
A) Effect of jaboticaba peel extract (JPE); B) Effect of pequi pulp aqueous extract (PAE). Total RNA was extracted and mRNA levels were assessed by real time polymerase chain reaction (RT-PCR). Data are expressed as mean ± standard deviation. * $p < 0.05$ versus control, # $p < 0.05$ versus H_2O_2 stressed cells, by Newman-Keuls test. Source: Authors.

In Figure 4A, it is observed that subsequently to the H_2O_2 stress induction, CAT mRNA expression decreased ($p < 0.05$) compared to control. Even treating stressed cells with JPE, CAT mRNA expression significantly decreased ($p < 0.05$) compared to stress-induced control. On the other hand, cells not induced by oxidative stress and treated with JPE had a significant ($p < 0.05$) increase in CAT mRNA expression relative to control.

In Figure 4B, the same behavior can be observed in PAE-treated C2C12 cells for CAT mRNA expression. Such results demonstrate that the addition of extracts in the cell culture medium induced CAT mRNA expression. However, when the cells were stress-induced with H_2O_2, the cultures showed reduced gene expression, and other enzymes may have performed the peroxide detoxification. These results differed from those of Priftis et al. (2018) and Goutzourelas et al. (2015), who reported that 17 µg mL^{-1} polyphenols from coffee extract did not affect CAT expression, whereas 10 µg mL^{-1} polyphenols from grape pomace extract decreased expression in C2C12 cells, respectively.

Regarding the GST enzyme, it can be seen in Figure 5 that no difference ($p > 0.05$) was found in mRNA expression between control and cells stressed with H_2O_2. However, increased gene expression ($p < 0.05$) was observed in stressed and JPE-treated cells compared to stressed cells without JPE addition.

Figure 5. Extracts effect on glutathione-s-transferase (GST) mRNA expression in C2C12 muscle cells without stress (control) and with hydrogen peroxide (H_2O_2) stress induction.

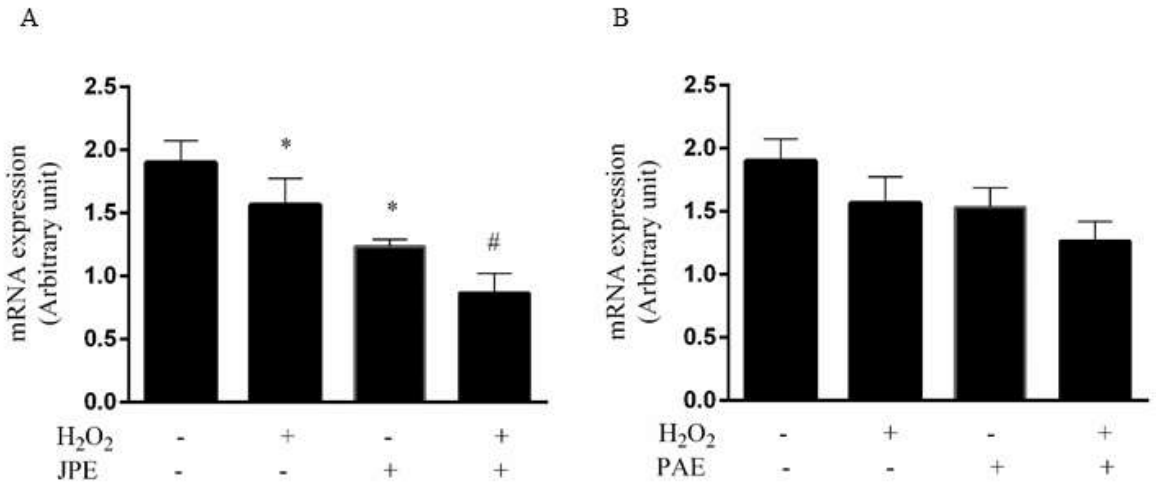


A) Effect of jaboticaba peel extract (JPE); B) Effect of pequi pulp aqueous extract (PAE). Total RNA was extracted and mRNA levels were assessed by real time polymerase chain reaction (RT-PCR). Data are expressed as mean ± standard deviation. * $p < 0.05$ versus control, # $p < 0.05$ versus H_2O_2 stressed cells, by Newman-Keuls test. Source: Authors.

In Figure 5A, increased gene expression was also observed with JPE treatment ($p < 0.05$) in non-stressed cells compared to control. In Figure 5B, the same behavior can be observed for PAE-treated C2C12 cells for GST mRNA expression. A significant effect of JPE and PAE treatments is noted when analyzing GST expression, which is a phase II metabolic enzyme responsible for detoxifying xenobiotics and active metabolites that may damage cells and tissues (Yang, Noh, Han, & Kim, 2010). GST enzyme is involved in detoxification by catalyzing glutathione conjugation and is considered one of the major enzymes associated with the elimination of peroxides formed during metabolism (Shih, Yeh, & Yen, 2007). Similar to the results of the present study, Priftis et al. (2018) described the antioxidant action of polyphenols from coffee extracts, which increased GST mRNA expression in C2C12 cells.

The GPX enzyme, discovered by Mills in mammalian tissues (Mills, 1959), protects cells from damage caused by lipid peroxide and H_2O_2. GPX1 is the most abundant selenoperoxidase and is ubiquitously expressed in almost all tissues (Xianyong et al., 2017). In Figure 6, it can be seen the JPE and PAE extracts effects on GPX1 mRNA expression in C2C12 muscle cells.

Figure 6. Extracts effect on glutathione peroxidase 1 (GPX1) mRNA expression in C2C12 muscle cells without stress (control) and with hydrogen peroxide (H_2O_2) stress induction.



A) Effect of jaboticaba peel extract (JPE); B) Effect of pequi pulp aqueous extract (PAE). Total RNA was extracted and mRNA levels were assessed by real time polymerase chain reaction (RT-PCR). Data are expressed as mean ± standard deviation. * $p < 0.05$ versus control, # $p < 0.05$ versus H_2O_2 stressed cells, by Newman-Keuls test. Source: Authors.

Figure 6A shows that after stress induction with H_2O_2, GPX1 mRNA expression was lower than control ($p < 0.05$). Reduction in gene expression ($p < 0.05$) was also observed in stressed and JPE-treated cells compared to stressed cells without JPE addition, as well as in JPE-treated cells compared to control without stress ($p < 0.05$). For PAE, there was no difference on GPX1 gene expression between treatments ($p > 0.05$) (Figure 6B).

The detoxification mechanism used by both GPX1 and GST enzymes is a conjugation with glutathione (GSH) (Goutzourelas et al., 2015). The GSH, a tripeptide composed of glycine, cysteine and glutamic acid, is one of the most critical antioxidant molecules in cells and is involved in detoxification of several xenobiotics and ROS. In the human organism, there are three major GSH systems: the GSH/GPX, which isolates the H_2O_2 produced during cellular metabolism; the GSH/GST, which combines GSH with xenobiotics for their detoxification (Aquilano, Baldelli, & Ciriolo, 2014); and the GSH/glutarredoxin (Grx), which controls the cellular redox environment (Lu & Holmgren, 2014). The JPE treatment increased GST mRNA expression by 7-fold compared to the control, which suggests that glutathione was conjugated with GST, decreasing GPX mRNA expression, and supposedly there was no GSH for conjugation. The PAE treatment increased GST mRNA expression by 3-fold compared to control, which suggests that GSH/GST system was also used; however, as the increase in GST expression was not as high as the treatment with JPE, it did not statistically influence GPX1 expression. Such differences in JPE and PAE effects may be attributed to differences in extracts chemical compositions.

4. Conclusion

This study has shown that treatments with jaboticaba peel and pequi extracts have an effect on CAT, GPX1 and GST genes expression. The extracts beneficial effects may be due to the synergistic activities of their bioactive compounds. Further studies on the regulation mechanisms of antioxidant defenses are needed to better understand and elucidate muscle disorders, in which reactive oxygen species play an important role.

For future researches, assays that measure products such as oxidized DNA bases, protein carbonyls, isoprostanes and 3-nitrotyrosine are recommended to further reinforce the findings and provide a clearer picture of the redox changes that are occurring as a result of the various treatments. Finally, the present study reinforces the use of these extracts as natural food colorings, being a field for in vivo tests.

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