

Cytoprotective Effects of Jaboticaba (*Plinia peruviana*, Poir. Govaerts) Fruit Peel Extracts against H₂O₂-Induced Oxidative Stress

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Pitz HS et al. *Reactive Oxygen Species* 4(11):362–371, 2017; ©2017 Cell Med Press

<http://dx.doi.org/10.20455/ros.2017.859>

(Received: June 30, 2017; Revised: July 18, 2017; Accepted: July 19, 2017)

ABSTRACT | Oxidative stress is a result of disturbance between the production of reactive oxygen species (ROS) and antioxidants defenses. Plant secondary metabolites can be used as exogenous antioxidants to prevent human oxidative stress-related diseases. This study aimed to determine the antioxidant and cytoprotective effects of jaboticaba fruit peel hydroalcoholic extracts against H₂O₂-induced oxidative stress in a murine fibroblast (L929 cell line) model. Ultra-high pressure hydroalcoholic extracts (50% ethanol, UP) were obtained over 4 h, with 1 h interval between collections. UP 3 h and UP 4 h extracts presented the highest and the lowest phenolic and flavonoid contents, respectively. Antioxidant capacity showed that 1 mg/ml extracts inhibited 90% of DPPH radical activity and UP 1 h extract had significant ($p < 0.05$) higher scavenging activity than other extracts at this concentration. After 24 h of exposure to UP 4 h extracts, cell viability was significantly increased when compared to control, UP 1 h, and UP 2 h extracts. When cells were previously treated with UP hydroalcoholic extracts followed by H₂O₂ exposure, all extracts at 0.05 and 0.1 mg/ml exhibited significant ($p < 0.05$) cytoprotective effects in relation to control. Results demonstrated that jaboticaba fruit peel extracts are antioxidants and exert cytoprotective effects under H₂O₂-induced oxidative stress.

KEYWORDS | Antioxidant; Cytoprotection; Jaboticaba; Oxidative stress; Phytochemicals

ABBREVIATIONS | CA, carnositic acid; CS, carnositic; DMEM, Dulbecco's modified Eagle medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; UP, ultra-high pressure

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1. INTRODUCTION

Oxidative stress is a result of disturbance between production of reactive oxygen species (ROS) and antioxidants defenses. Excessive ROS production causes oxidative damage to biomolecules such as proteins, lipids, and nucleic acids. Consequences of this process may cause cell dysfunction and altered behavior or even cell death [1].

Prevention of oxidative stress is mediated by antioxidants molecules. These substances delay, prevent, or remove the oxidative damage to a target molecule [2]. Endogenous antioxidants are classified as enzymatic and non-enzymatic. Superoxide dismutase, catalase, and glutathione peroxidases are examples of enzymatic antioxidants which prevent formation of ROS [3, 4], while glutathione, ubiquinone, urate, and lipoic acid are non-enzymatic defenses [5].

Plant's non-enzymatic antioxidant systems have evolved efficiently and most of those molecules result from the plant secondary metabolism, being required for normal growth, development, and plant defense. However, plant metabolism is susceptible to biotic and abiotic stress factors such as high light intensity, heat, drought, anoxic conditions, and pathogen attacks that increase ROS production. As a result of stress conditions, plants are able to synthesize a broad range of antioxidant molecules [6–8]. Phenolic compounds are examples of secondary metabolites which exhibit increased contents in plant tissues under stress conditions [9].

The intake of polyphenols has been related to preventive medicine helping to decrease the risk of developing cancer, neurodegenerative and cardiovas-

cular diseases in human populations and animal's models [10–12]. Jaboticaba (*Plinia peruviana*, Poir. Govaerts) is a native fruit tree to Brazil and its fruits have been claimed to be a rich source of phenolic compounds such as anthocyanins, flavonoids, and ellagitannins [13] with a known antioxidant activity [14]. In this sense, this study aimed to determine the antioxidant and cytoprotective effects of jaboticaba fruit peel extracts, obtained through a high-pressure technique during different times, against H₂O₂-induced oxidative stress in murine L929 fibroblasts.

2. MATERIAL AND METHODS

2.1. Plant Material Collection and Extraction

Fruits of *P. peruviana* were collected from a backyard format planting system during the harvest season (Spring, 2014) in Guaxupé, Minas Gerais, southeastern Brazil (21° 18' 19" S, 46° 42' 46" W, 829 m above sea level). The plant was authenticated by Dr. Marcos Sobral, and a voucher specimen (FLOR 55902) was preserved at the herbarium Flor (Department of Botany, Federal University of Santa Catarina, Florianópolis, Brazil).

The fruit peels of jaboticaba were previously sanitized with tap water, lyophilized, and powdered using an electric grinder. The dried and powdered biomass was added (1:10 w/v) to 50% ethanol solution (v/v, pH 3.6). The mixture was submitted to a home-made ultra-high pressure (7 to 10 kg/cm²) extractor at room temperature during different times as follows: 1 h (UP 1 h extract), 2 h (UP 2 h extract),

TABLE 1. Total phenolic and flavonoid contents (mg equivalents/g) of the UP hydroalcoholic extracts of jaboticaba peels measured at 1, 2, 3, and 4 h

	UP 1 h	UP 2 h	UP 3 h	UP 4 h
Phenolic content (mg galic acid equivalent/g)	20.44 ± 0.95 a	17.85 ± 0.08 a	32.75 ± 0.79 b	14.1 ± 0.55 c
Flavonoid content (mg quercetin equivalent/g)	6.29 ± 0.07 a	5.13 ± 0 b	8.7 ± 0.16 c	3.84 ± 0.03 d

Note: Values are shown as mean ± SD (n = 3). Distinct lowercase letters indicate meaningful (p < 0.05) differences between the UP extracts in the same line.

3 h (UP 3 h extract), and 4 h (UP 4 h extract). At the end of each of the collection times, all extracts were recovered by filtration on cellulose membrane under vacuum.

2.2. Phytochemical Analysis

2.2.1. Total Phenolics Content

The total phenolic content of extracts was measured spectrophotometrically [15]. Briefly, extracts were diluted in 50% ethanol solution, pH 3.6 (1:10 v/v). Subsequently, 1 ml of each extract previously diluted was added to 5 ml 95% methanol solution. After this second dilution, a sample (1 ml) was added to 1 ml 95 % ethanol solution, 5 ml distilled water, and 0.5 ml Folin-Ciocalteu's reagent and incubated for 7 min. After incubation, 1 ml 5 % sodium carbonate solution (w/v) was added and kept in the darkness at room temperature for 1 h. The absorbance was measured at 725 nm, using a UV-Vis spectrophotometer (BEL LGS 53, BEL Engineering, Monza, Italy). The total phenolic compounds were quantified using a standard curve of gallic acid (10–100 µg/ml, $y = 0.0076x$, $r^2 = 0.99$). The results were expressed as mg gallic acid equivalents/g dry weight of jaboticaba extract.

2.2.2. Total Flavonoids Content

The total amount of flavonoids was determined based on the aluminum chloride colorimetric method [16]. The extracts were diluted (1:10 v/v) in 50 % ethanol (pH 3.6) and 0.5 ml of diluted extracts was added to 2.5 ml ethanol and 0.5 ml 2% aluminum chloride in methanol, following incubation for 1 h. The absorbance was measured at 420 nm. The quantification of total flavonoids was carried out using a

quercetin standard curve (10–200 µg/ml, $y = 0.0089x$, $r^2 = 0.99$). The results were expressed as mg quercetin equivalents/g dry weight of jaboticaba extract.

2.3. Antioxidant Activity (DPPH Assay)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is a chemical method that measures the capacity of a compound to scavenge free radicals based on the decrease in absorbance during the reaction [17]. For measuring the antioxidant activity of extracts, a DPPH stock solution (3.16 mg DPPH/ml methanol) was prepared and further 1:100 diluted in 80% methanol (v/v). The absorbance of the DPPH solution should be around 0.5 and 0.6. Jaboticaba hydroalcoholic extracts were diluted in 50% ethanol (pH 3.6) at concentrations of 0.001, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, and 1 mg/ml (dry weight extract). Subsequently, extracts were added to DPPH methanolic solution at a ratio of 1:30 (v/v). The antioxidant activity of extracts was measured spectrophotometrically at 515 nm, after 30 min incubation in the dark, at room temperature. The percentage of inhibition of DPPH radical was calculated by the equation below:

Inhibition DPPH (%) = $[(\text{Abs DPPH}/80\% \text{ methanol solution} - \text{Abs UP extracts}) \div (\text{Abs DPPH}/80\% \text{ methanol solution})] \times 100$; where Abs denotes absorbance at 515 nm.

2.4. Cell Viability Assay Using L929 Fibroblasts

L929 mouse fibroblast cells were inoculated at a density of 5×10^3 cells/well into a 96-well plate in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C, in a humidified 5% CO₂ atmosphere, overnight. After incubation, DMEM was re-

TABLE 2. Antioxidant activity of UP extracts determined by the DPPH assay

	UP 1 h (%)	Trolox Eq	UP 2 h (%)	Trolox Eq	UP 3 h (%)	Trolox Eq	UP 4 h (%)	Trolox Eq
0.001 mg/ml	6.63 ± 1.82 Aa	0.047	5.82 ± 0.94 Aa	0.041	11.23 ± 1.44 ABb	0.08	7.04 ± 0.33 Aa	0.05
0.01 mg/ml	8.23 ± 1.12 Ba	0.058	4.08 ± 0.58 Ab	0.029	8.42 ± 2.64 Aa	0.06	7.19 ± 1.35 Aab	0.051
0.05 mg/ml	17.68 ± 3.68 Ca	0.125	10.17 ± 2.61 ABb	0.072	14.64 ± 0.95 Bab	0.104	13.93 ± 2.2 Bab	0.099
0.1 mg/ml	25.14 ± 2.74 Da	0.178	16.57 ± 5.72 Ba	0.117	22.18 ± 1.12 Ca	0.157	23 ± 0.68 Ca	0.163
0.2 mg/ml	39.72 ± 2.51 Ea	0.282	32.93 ± 8.72 Ca	0.233	36.78 ± 1.62 Da	0.261	34.4 ± 0.66 Da	0.244
0.4 mg/ml	65.83 ± 1.47 Fa	0.467	52.69 ± 6.45 Db	0.374	62.02 ± 1.48 Ea	0.44	58.36 ± 0.89 Eab	0.414
0.8 mg/ml	89.45 ± 1.47 Ga	0.634	82.27 ± 2.9 Eb	0.583	86.74 ± 0.26 Fa	0.615	84.7 ± 0.24 Fb	0.6
1.0 mg/ml	93.51 ± 0.66 Ga	0.663	90.43 ± 0.28 Fb	0.641	90.86 ± 0.18 Gb	0.644	89.96 ± 0.06 Gb	0.638

Note: Values are shown as mean ± SD (n = 3). Trolox Eq, trolox equivalent (mM). Distinct capital letters indicate significant differences ($p < 0.05$) for the concentrations of the same extract. Distinct lowercase letters indicate meaningful ($p < 0.05$) differences between the extracts at the same concentrations.

placed by DMEM 10% FBS containing 0.001, 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, and 1 mg/ml of each extract (dry weight), except in control where the culture medium was replaced by fresh DMEM. Cells were incubated for 24 h and 48 h, at 37°C, in a humidified 5% CO₂ atmosphere. Afterwards, the culture medium was replaced by 100 µl of fresh DMEM along with 10 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT solution, 5 mg/ml in phosphate-buffered saline, PBS) per well and incubated in the dark (3 h, 37°C, humidified 5% CO₂ atmosphere). Subsequently, 85 µl culture medium were removed and 50 µl of dimethyl sulfoxide (DMSO) were added onto each well. After solubilization of the formazan crystals, the absorbance was determined with an ELISA plate reader at 540 nm. The percentage of cell viability was calculated and compared to control (100% viability).

2.5. H₂O₂-Induced Oxidative Stress in L929 Fibroblasts and Evaluation of Cell Survival

Hydrogen peroxide was used for induction of oxidative stress as described by Balekar et al. [18] and

Ponnusamy et al. [19]. A curve with H₂O₂ concentrations (0.0625, 0.125, 0.25, 0.5, and 1.0 mM) was built to determine the H₂O₂ dose which decreases cell viability by 80 % after 24 h of exposure through MTT assay. The chosen concentration was 1.0 mM H₂O₂. Subsequently, fibroblast L929 cells were inoculated (5×10^3 cells/well) into a 96-well plate containing DMEM supplemented with 10% FBS and incubated overnight at 37°C, in a humidified 5% CO₂ atmosphere. After incubation, DMEM 10 % FBS containing 0.001, 0.01, 0.05, 0.1, and 0.2 mg/ml of each extract (dry weight) was used to treat cells in different times as follows: (1) cells were treated for 24 h following by 1.0 mM H₂O₂ exposure for 3 h; (2) cells exposure concomitantly to extracts and 1.0 mM H₂O₂ for 24 h; and (3) 1.0 mM H₂O₂ exposure for 3 h following by cells treatment with each extract for 24 h. Evaluation of cell survival was performed using the MTT assay as described above.

2.6. Statistical Analysis

Data were collected and summarized, followed by univariate statistical analysis using the one-way

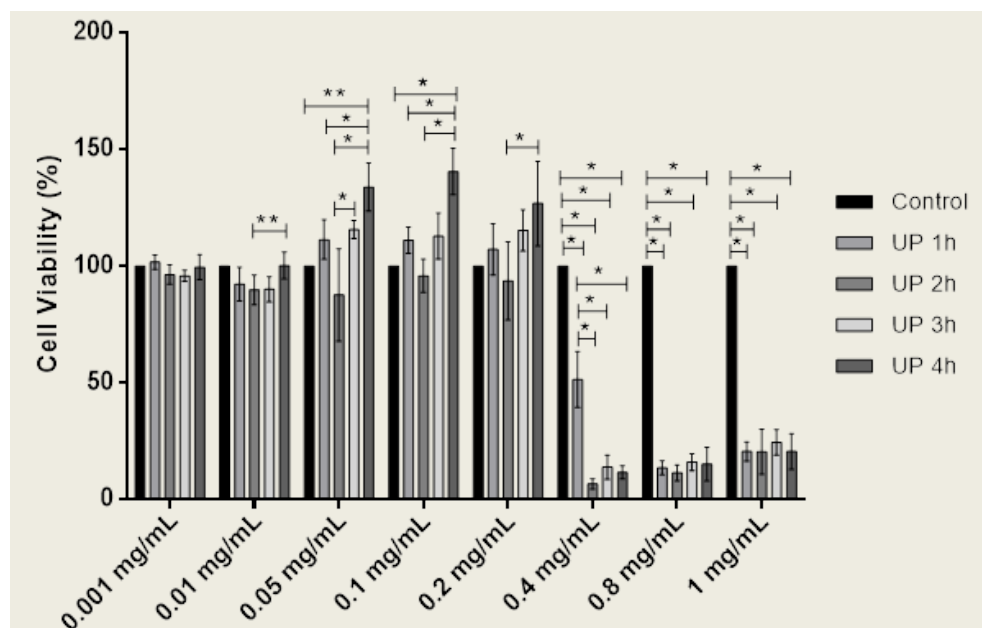


FIGURE 1. Percentage of L929 fibroblast cell survival following treatment with UP extracts after 24 h. Data are expressed as mean \pm SD ($n = 6$). *, $p < 0.01$; **, $p < 0.05$ vs control and different UP extracts at the same concentration.

ANOVA and Tukey's test. P values lower than 0.05 were considered to be statistically significant. The values were expressed as mean \pm SD or median as indicated in figures legends.

3. RESULTS

3.1. Total Phenolic and Flavonoid Contents of UP Extracts

UP 3 h extract showed the highest amount ($p < 0.05$) of total phenolic compounds, significantly differing from the following extraction, i.e., UP 4 h, which revealed the lower concentration (Table 1). Similarly, the recovery of flavonoids in the hydroalcoholic extracts showed to be highest ($p < 0.05$) after 3 h extraction, with lower amounts after UP 4 h (Table 1).

3.2. Antioxidant Activity

All the tested extracts presented inhibitory activity of radical DPPH being higher than 50% at concentration 0.4 mg/ml. Interestingly, at this concentration

UP 2 h extract showed significantly ($p < 0.05$) lower antioxidant activity than UP 1 h and UP 3 h extracts. DPPH scavenging rate increased in a dose-dependent manner until 1 mg/ml, where UP 1 h extract showed the best DPPH scavenging activity which was significantly higher than other extracts. Maximum radical inhibition at 1 mg/ml concentration of extracts was equivalent to about 0.6 mM of trolox, an antioxidant water-soluble analog of vitamin E (Table 2).

3.3. Cell Viability

After 24 h of exposure to UP 1 h, UP 3 h, and UP 4 h extracts at 0.05 and 0.1 mg/ml, an increased viability of L929 cells was observed, and UP 4 h extract significantly augmented that variable comparatively to control, UP 1 h, and UP 2 h treatments. Importantly, at higher concentrations, i.e., 0.4, 0.8, and 1 mg/ml, cells treated with all UP extracts showed decreased viability in a significant way in respect to control after 24 and 48 h of exposure (Figures 1 and 2). No significantly increased cell viability was detected after 48 h of exposure to the hydroalcoholic extracts of jaboticaba peels compared to control (Figure 2).

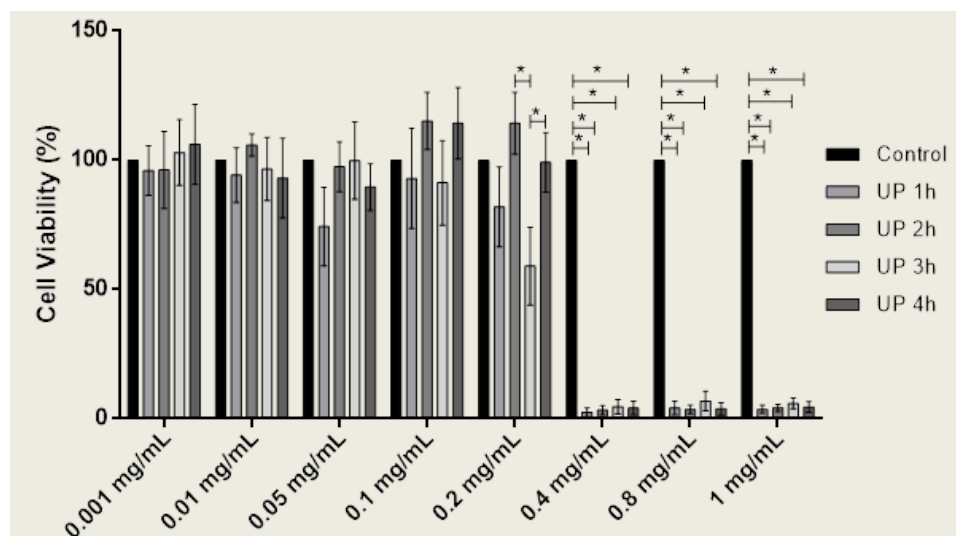


FIGURE 2. Percentage of survival of L929 fibroblast cells treated with UP extracts after 48 h. Data are expressed as a mean \pm SD ($n = 9$). *, $p < 0.01$ vs control and different UP extracts at the same concentration.

3.4. H₂O₂-Induced Oxidative Stress and Cell Survival

Cells exposed concomitantly to the hydroalcoholic extracts and H₂O₂, and cells exposed to extracts after H₂O₂-induced oxidative stress showed increased cell death. UP 3 h- and UP 4 h-treated cells presented significantly ($p < 0.05$) higher viability than control when exposed concomitantly and after H₂O₂, despite cell viability was lower than 20% (**Figure 3**). Preincubation with UP 1 h, UP 3 h, and UP 4 h hydroalcoholic extracts followed by H₂O₂ exposure led to significantly ($p < 0.05$) increased cell viability in comparison to control at 0.001 mg/ml concentration. At 0.01 mg/ml, UP 4 h extract increased ($p < 0.05$) L929 fibroblast viability when compared to control and other treatments. At 0.05 mg/ml, significantly increased cell viability was observed in UP 2 h, UP 3 h and UP 4 h when compared to control and UP 1 h extract. However, the highest cell viability was found at 0.1 mg/ml for all extracts which differed in a significant way from control.

4. DISCUSSION

Plant-derived phenolics are well-known as beneficial for human health due to their antioxidant activity.

These phytochemicals can act in different ways to protect cells from oxidative damage. The mechanisms of action include prevention of reactive species formation, scavenging radicals or repairing damaged target molecules [20].

The antioxidant activity of jaboticaba fruit peel is thought to be related to its content of phenolic compounds. However, these molecules are unstable and could easily suffer degradation. Therefore, some parameters such as temperature, exposure to O₂, and time of extraction should be monitored during extraction process to avoid loss of biological activity as a consequence of phenolic compound degradation [10]. In order to overcome such constraints, an alternative method to improve the recovery of phenolics from jaboticaba fruit peel was adopted, by grinding the biomass and extracting it under ultra-high pressure in the absence of O₂, at room temperature. A time series for collection of the extracts was set, with intervals of 1 h, over an experimental time of 4 h.

The sampling time for harvesting of the hydroalcoholic extracts showed to be relevant regarding the total amounts of phenolic and flavonoid compounds. Higher concentrations of those secondary metabolites were found in UP 3 h extract when compared with other extracts. On the other hand, longer extraction times, i.e., UP 4 h presented significantly lower contents of the target compounds. Taking into ac-

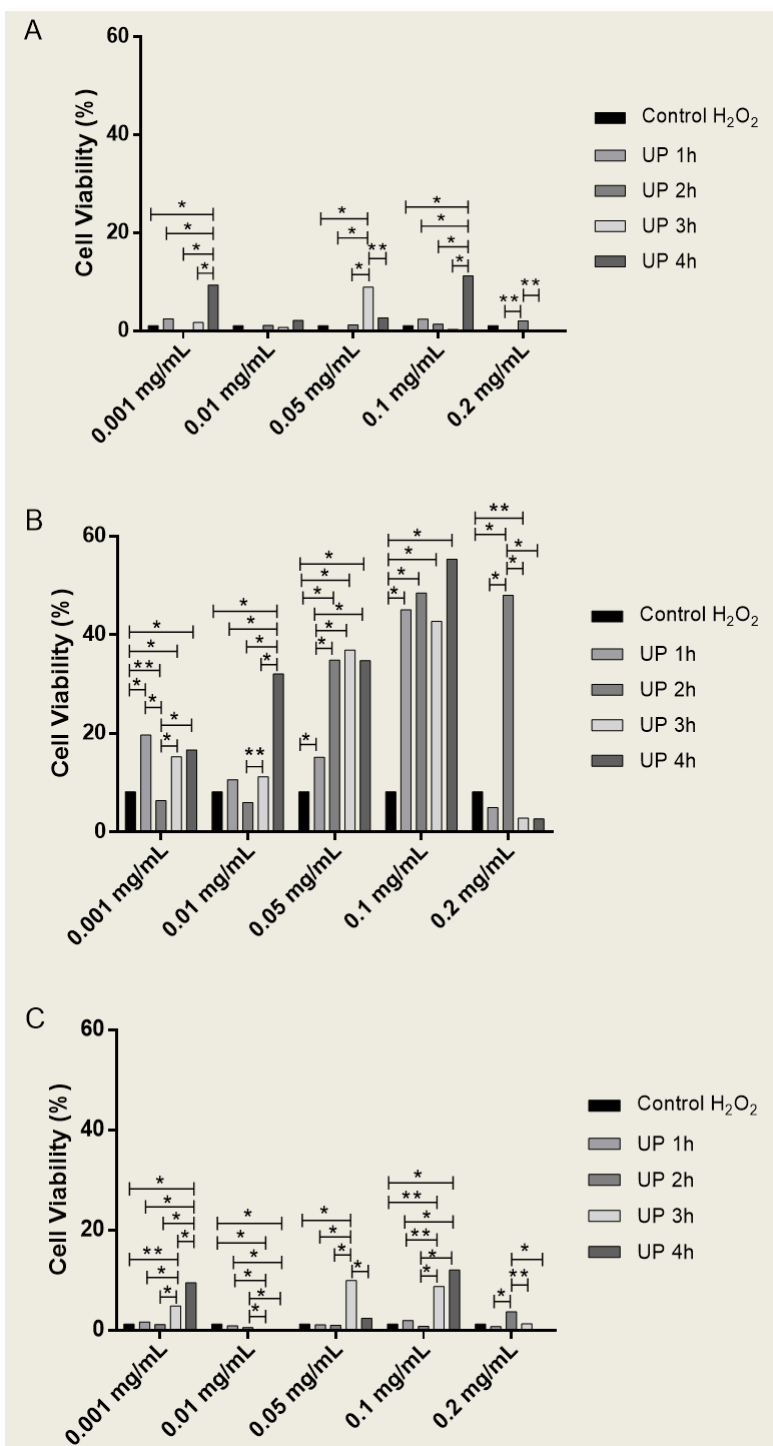


FIGURE 3. Viability (%) of L929 fibroblast cells treated with UP hydroalcoholic extracts after H₂O₂ exposure (A), before H₂O₂ exposure (B), and concomitant to H₂O₂ (C). *, p < 0.01 and **, p < 0.05 vs 1.0 mM H₂O₂ control and different UP extracts at the same concentration. Cell viability values ranged from 0 to 20% during treatments in A and C, and due to this variation data were expressed as median (n = 16).

count these findings, one could speculate that further extractions would afford even lower amounts due to the depletion of those secondary metabolites from the fruit peel biomass.

In a second series of experiments the scavenging properties of the UP extracts were tested *in vitro* by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The scavenging of DPPH radical is mediated by an antioxidant compound through hydrogen atom donation or one electron transfer to the radical, forming a stable compound [21, 22]. Although the UP extracts revealed an interesting antioxidant activity, at higher concentrations they were harmful to L929 fibroblasts in culture. At concentrations of 0.4 mg/ml or above, UP extracts were cytotoxic to L929 cells after 24 and 48 h exposure. This effect could be explained by a possible pro-oxidant property exerted by high concentrations of antioxidants. Indeed, similarly to vitamin C, phenolics are capable of converting iron and copper ions into their reduction forms which react with hydrogen peroxide to produce the highly toxic hydroxyl radicals [23–25]. Factors that contribute to turning antioxidants into pro-oxidants include the concentrations of the antioxidants, nature of neighboring molecules [26], and availability of transition metals [24].

All UP extracts at concentrations between 0.001 and 0.2 mg/ml were investigated regarding their protective effect using an *in vitro* oxidative stress model. For that, L929 fibroblast cells were exposed to H₂O₂ for 3 h, and the UP extracts were added to the culture medium before or after the H₂O₂ exposure. In addition, cells were also concomitantly treated with UP extracts and H₂O₂ for 24 h. Hydrogen peroxide-induced oxidative stress before and concomitant with the extracts was lethal for L929 cells. H₂O₂ is a small uncharged molecule that could diffuse through membrane cells easily [27]. This rapid transport to inside the cell could be responsible for the deleterious effect of H₂O₂ that acted faster than the cytoprotective phytochemicals added to the culture medium.

Extracts added before H₂O₂ exposure were effective in protecting L929 cells from death, mainly with the UP 4 h extract. Preincubation with UP extracts led to metabolic changes that resulted in cytoprotective effects against severe stress caused by H₂O₂. Phytochemicals can promote mild stress induction that elicits adaptive beneficial responses, thereby increasing protection against a further oxidative challenge. This process is known as hormesis [28–30]

and might corroborate to explain the results herein described.

Carvalho et al. [31] studied the antioxidant phenolic diterpenes carnosil acid (CA) and carnosol (CS) and observed that CS was able to protect cells against tert-butyl hydroperoxide (t-BOOH)-induced death after phytochemical preincubation. Antioxidant defenses were measured and both CA and CS increased the levels of the reduced form of glutathione (GSH), heme oxygenase-1, and glutamate cysteine ligase modulatory subunit by changing cell redox stress. The authors found that disturbance of cellular redox state was not promoted by induction of ROS. Treatment with CA and CS increased transcription activity of Nrf2, a factor responsible for transcription of genes encoding stress-responsive and cytoprotective enzymes and related proteins.

Although UP extracts at 0.2 mg/ml maintained the cell viability after 24 and 48 h of exposure, preincubation with this concentration followed by H₂O₂ treatment showed to be cytotoxic for UP 1 h, 3 h, and 4 h extracts. It has been hypothesized that the subsequent incubation with H₂O₂ shall potentiate possible pro-oxidant effects caused by the UP extracts as discussed above.

Evaluation of the effectiveness of UP extracts regarding their antioxidant activity was performed *in vitro* using the L929 murine fibroblast cell line. Fibroblasts are skin cells and play an important role during wound healing process. Secretion of ROS is mediated by neutrophils and monocytes during inflammation phase in normal wound healing and excessive production of these substances could impair proliferation and migration of dermal fibroblasts during healing [27]. Taking into account the results herein described, it seems plausible that jaboticaba peel hydroalcoholic extract is an important source of bioactive polyphenolic compounds able to inhibit the oxidative stress caused by H₂O₂ to fibroblasts. This way, the UP extract might be thought as an interesting candidate to study with pre-clinical assays regarding its wound healing effect.

5. CONCLUSION

The extraction time of 3 h allowed the better yield of phenolic and flavonoid compounds from jaboticaba fruit peels. Even though UP 4 h extract presented the lowest phenolic and flavonoid contents compared to

other extracts, it increased the cell viability when compared to control. All the hydroalcoholic extracts studied afforded cytoprotective effect to L929 fibroblasts against H₂O₂, revealing jaboticaba fruit peels as a promise source of bioactive compounds with potential biotechnological applications in promoting human health.

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