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Evaluation of the Antioxidant, Photoprotective and Wound Healing Capacity of *Guazuma ulmifolia* lam. Extracts in L-929 Cells Exposed to UV-A and UV-B Irradiation

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The UV irradiation causes the generation of reactive oxygen species in the skin that mediate oxidative damage, favoring photoaging and skin cancer. Photoaging can be mitigated with the use of plants with antioxidant capacity. This study sought to evaluate the photoprotection capacity of two extracts of *Guazuma ulmifolia* Lam., aqueous (G1) and acetonic (G2), on UV-A and UV-B irradiated L-929 cells, besides the wound healing capacity. In the antioxidant evaluation, it was found that the extracts have great antioxidant potential. In the irradiation tests, G2 increased cell viability by 19.99%. Furthermore, both extracts have shown wound healing capacity. There was a significant restoration of the superoxide dismutase and catalase enzymatic activity by G2 and through ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS/MS), two antioxidant compounds were identified: (epi)catechin and procyanidin. The results showed that the extracts are promising antioxidants, and may collaborate in wound healing, with emphasis to G2 which protected cells from UV-A irradiation.

Keywords: photoaging, mutamba, reactive oxygen species, ultraviolet radiation

Introduction

The sun is indispensable for life on Earth; however, it is a fact that the sun's rays can be harmful to human beings. The use of some sort of artificial lights, the increase in human life expectancy and the degradation of the ozone layer leads people to a greater exposure to UV radiation.[1] Climate change, which currently manifests itself in the form of global warming, can affect the intensity of UV radiation through a change in the thickness of the ozone layer. Ozone is an effective absorber of solar UV radiation and changes in its layer allow higher levels of UV radiation to reach people's skin.[2] The skin is the body part that is most susceptible to sun radiation and environmental pollution. Thus, a disharmony between the amount of reactive oxygen species (ROS) and its capture by the antioxidant system of the human body results in an oxidative stress, causing damage to macromolecules, degradation of the cellular structure and compromising the functioning of the cell.[3] The main generators of ROS (nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) and oxidative phosphorylation) are activated by UV radiation.[4]

The solar radiation and their ultraviolet light can be classified as: UV-A (320-400 nm), a ray with lower energy value but more abundant that reaches deeper layers of the skin; UV-B (280-320 nm) that reaches superficial parts of the skin, and UV-C (200-280 nm) which is almost completely prevented from reaching the ground by the ozone layer. UV-A and UV-B are the main stimulators of skin damage such as photoaging and cancer.[5] UV-A is the mainly responsible radiation for immediate tanning or darkening of

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the skin due to excess production of melanin in the epidermis, suppression of immunologic functions, and even necrosis of endothelial cells and damage of dermal blood vessels.[6] Now, UV-B radiation acts especially on the basal cells of the epidermis, causing burns which can lead to skin cancer.[6]

Natural products with antioxidant potential are extremely important because they may be able to reduce the occurrence of skin aging caused by the sun.[7] Research on photochemicals with antioxidant and photoprotective capacity has gained more and more strength in the scientific community, as well as in the cosmetic and cosmeceutical sectors, aiming to improve the quality and efficiency of sunscreens.[8] In addition, these natural compounds have been used to reduce and even replace synthetic molecules present in sunscreens.[8] Some examples of recent studies in the use of plants with antioxidant and photoprotective potential are: the use of citrus fruit residues,[9] seaweed,[10] pineapple peel,[11] *Baccharis dracunculifolia*,[12] banana peel,[13] *Inga edulis*,[14] *Cyclopia* sp.[15] and *Kaempferia galanga* L.[16]

Guazuma ulmifolia Lam. (Malvaceae), also known as mutamba, is a common tree species in Latin America, especially in Mexico and Brazil. Hör *et al*.[17] and Felipe *et al*.[18] identified phenolic compounds in *Guazuma* extracts such as epicatechin and procyanidin. Phenolic compounds are potent antioxidants and even safer than synthetic antioxidants.[19] Different mutamba parts have shown potential as a high antioxidant activity,[20] however, the photoprotective potential of *Guazuma ulmifolia* Lam. in L-929 cells has not yet been studied.

The present study evaluated the photoprotective effects of two extracts of *G. ulmifolia*, an aqueous (G1) and acetonic (G2) extract, on UV-A and UV-B irradiated L-929 cells and the wound healing capacity. The results obtained could contribute to the improvement in the efficiency of sunscreens, mainly in the antioxidant action, preventing photoaging as well as the reduction of synthetic molecules commonly present in sunscreens, hence making this product more natural.

Experimental

Chemicals

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Life Technologies/Gibco Laboratories, Grand Island/NY, USA,). Ethanol, glacial acetic, acid acetone, methanol, formaldehyde, calcium chloride and dimethyl sulfoxide (DMSO) were purchased from Synth (Diadema, Brazil). Phosphate buffered saline (PBS), hank's balanced salt solution (HBSS), *N*-acetylcysteine (Nac), and quercetin (QT) were purchased from Sigma-Aldrich (St. Louis, USA) and 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^•$) neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) were purchased from Inlab® (São Paulo, Brazil).

Plant material

Barks of *Guazuma ulmifolia* Lam. were collected in Ibiporã, State of Paraná, Brazil (23°18'15.2"S; 050°58'32,7"W; 396 m of altitude; Garmin v.2.24). The botanical material was registered by the Brazilian Biodiversity System (SisGen) with the code: ADA98FE.

The extraction procedure was performed using 1000 g of air-dried bark in water (G1) and acetone:water (7:3) (G2) adopting the turbo-extraction using an Ultra-turrax® UTC115KT (Ika Works, San Diego, USA); 20 min; temperature ≤ 40 °C.[21] The crude extracts were filtered and evaporated under reduced pressure and then lyophilized using the Equipment Christ Alpha 1-4 LSC (Osterode am Harz , Germany, Martin Christ Freeze Dryers) and the following parameters: condenser temperature of –55 ºC and drying time of 24 h.

Analysis by UHPLC-HRMS/MS

The characterization of the extracts was performed by ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS/MS) using the following parameters: ultra-efficiency liquid chromatography instrument Shimadzu Nexera X2 (International Equipment Trading Ltd., Lake County, USA), controller CBM-20A, pumps LC-30AD, CTO-30A column oven and sampler automatic SIL-30AC. Column C18 (75 × 2.0 mm internal diameter; 1.6 µm Shim-pack XR-ODS III). Injection volume: 3 µL. Gradient: A (H_2O) and B (acetonitrile): 5% B 0-2 min, 30% B 2-3 min, 95% B 3-10 min, maintained at 95% B 10-14 min, and 5% B 14-15 min at 40 °C. High resolution mass spectrometry Bruker IMPACT II spectrometer, ionization source electrospray, analyzer: quadrupole-time of flight and detector: multichannel plate. Capillary voltage: 4500 V, calibrant: sodium formate (10 µM). Gas: 8 L min^{-1} at 200 °C, pressure: 4 bar. Collision gas: argon and collision energy: 10-45 eV. Mass range: *m/z* 50-1950; acquisition rate: 5 spectra *per* second. MS/MS auto-scan shredding.

Antioxidant capacity

The following methods were used to evaluate the antioxidant capacity of extracts of *Guazuma ulmifolia* Lam.:

free radical-scavenging ($DPPH^•$) as previously described by Brand-Williams *et al.*,[22] ferric reducing antioxidant power (FRAP), as described by Benzie and Strain;[23] and $ABTS^{•+}$ (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay, based on a method developed by Miller *et al.*,[24] with modifications. The extract was solubilized with methanol and diluted to the concentrations for the $DPPH^•$ assay: 125, 62.5, 31.25, 15.62, 7.81, 3.90 and 1.95 μg mL^{-1}. For the FRAP, the extract was solubilized with ethyl alcohol and diluted to the following final concentrations: 20.83, 10.41, 5.20 and 2.60 μg mL^{-1} and for the $ABTS^{•+}$ the extract was solubilized with ethyl alcohol and diluted to the concentrations: 8.45, 4.22, 2.11 and 1.05 μg mL^{-1}. A microplate spectrophotometer (Bio Tek Power Wave XS, Winooski, USA) was used for the analysis of the results at 517 nm. It was also tested scavenging activity against superoxide radicals using the xanthine/luminol/xanthine oxidase (XOD) system as described by Girotti *et al.*[25] A plate reader Spectramax L. (Molecular Devices, San Jose, USA) was used and the extract was solubilized in 50% ethyl alcohol. The concentrations used were: 14.3, 7.4, 3.7, 1.8, 0.9, 0.4, and 0.2 μg mL^{-1}. The concentration responsible for capturing 50% of free radicals (EC_{50}) was considered in the methodologies $DPPH^•$ and XOD. In the methods FRAP and $ABTS^{•+}$, the values are equivalent to millimolar Trolox *per* gram of sample.

Photoprotective potential

Cell culture

The fibroblast cell line L-929 (clone NCTC 929, L CELL, L-929; ATCC® CCL1™, Manassas, USA), was given by Dr Maria José Vieira Fonseca (Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil). The following conditions were provided for cell culture: incubation at 37.0 °C and 5.0% CO_2.[26] Cell culture was carried out in DMEM containing penicillin and streptomycin; L-glutamine and FBS.

Cytotoxicity

Cytotoxicity was evaluated in L-929 fibroblasts using the neutral red cell (NR) viability assay.[27] The cells were treated with different concentrations of the G1 and G2 (125, 62.5, 31.25, 15.62, 7.81 and 3.90 μg mL^{-1}) previously diluted in DMSO (10,000 μg mL^{-1}) and incubated for 24 h in an oven at 37.0 ºC with 5.0% CO_2. Some wells were maintained only with DMEM, negative control (NC). A plate reader (Bio Tek Power Wave XS, Winooski, USA) at a wavelength of 540 nm was used. The CC_0 (concentration responsible for 0% cell inhibition) was determined by non-linear regression analysis.

In vitro irradiation

UV-A irradiation was performed using a Philips TLK 40W/10R lamp (AKARI Lâmpadas & Leds, São Paulo, Brazil). The energy dose was measured by a UV radiometer VLX-3W, Vilber Lourmat (Biotech-sl, Las Rozas, Spain) that was equipped with a UV-A detector applied at an intensity of 15 J cm^{-2} and UV-B irradiation was performed using a Philips TL40W/12RS (AKARI Lâmpadas & Leds, São Paulo, Brazil); applied at an intensity of 600 mJ cm^{-2}. For the irradiation assays, 24-well plates were used with 2.5×10^5 cells $well^{-1}$ and grown for 24 h. The G1 and G2 extracts were diluted first in 1% DMSO and then in DMEM to reach the final concentrations of 7.81, 3.90 and 1.95 μg mL^{-1}. For the pre-treatment, the extracts were in contact with the cells 1 h before irradiation. NC and UV-A/UV-B wells were treated only with DMEM. Before irradiation, the medium was changed to HBSS.[28] After incubation for 24 h, the cells were washed with PBS, treated with 100 µL of the NV solution, and incubated for 3 h. Subsequently, the fixative solution (1% $CaCl_2$ and 2% formaldehyde) was added and, finally, the ethanolic solution (50% ethanol/water solution and 30% glacial acetic acid). The plates were read in a plate reader BioTek Power Wave XS (BioTek® Instruments, Winooski, USA) and cell viability was determined considering the negative control.

Scratch assay

The wound healing capacity of L-929 cells were assessed using a scratch wound assay which measures the expansion of a cell population on surfaces, applying the methodology described by Balekar *et al.*,[29] with some modifications. L-929 cells were seeded into 24 well microplate at a density of 2.5×10^5 cells $well^{-1}$, and grown for 24 h. Afterwards, the cells were incubated for 6 h with 0.5% FBS. The wounds were made with a micropipette tip, wells were washed with PBS and treated for 0, 24, and 48 h with G1 and G2 in the concentrations of 7.81, 3.90 and 1.95 μg mL^{-1}. NC wells were treated only with DMEM. Cell migration was observed under an inverted microscope with phase contrast Olympus CKX41-5× magnification (Olympus Optical do Brasil Ltda, São Paulo, Brazil) and the obtained images were quantified by the software ImageJ (USA, National Institute of Mental Health).[30]

Endogenous antioxidant defenses: catalase, superoxide dismutase and glutathione reduced assays

L-929 cells were plated in a 6-well plate: 4×10^5 cells mL^{-1} cell lysates were prepared after 24 h of UV-A irradiation by scraping cells in ice-cold lysis buffer, followed by sonication in 4C15, Branson Ultrasonics (Sigma-Aldrich, Barueri, Brazil) on ice for 60 s and

centrifugation at 14,000 for 10 min at 4 °C. The supernatant was collected and then quantified by Bradford.

The samples were stored in a freezer at –80 °C until they were evaluated. The catalase activity (CAT) assay is based on the reduction of H_2O_2. The behavior of the extracts was evaluated for 5 min in a Shimadzu reader (UV-1700, Shimadzu, Barueri, Brazil). A CAT fit curve was performed and its activity was given in units *per* mg of protein.[31]

Reduced glutathione (GSH) levels were determined using a 96-well black plate to which lysate (10 µL), *o*-phthaldehyde (OPT; 1 mg mL^{-1} in methanol) and 180 µL of sodium phosphate buffer were added. After 15 min of incubation, reads were performed on a spectrofluorometer (Victor® X3, PerkinElmer, Waltham, USA). The results were expressed in µg GSH *per* mg of protein where a calibration curve with GSH was used.[32]

For the superoxide dismutase (SOD) activity assay, a reaction mixture composed of cell lysate, pyrogallol and Tris-HCl buffer was used. The behavior was evaluated at 420 nm in spectrum (UV-1700, Shimadzu, Barueri, Brazil) for 2 min. Results were obtained in units *per* mg of protein using a calibration curve made with SOD standard.[33]

Statistical analysis

The results are expressed as the mean ± standard deviation (SD) of at least three independent tests. Statistical analyzes were performed using the GraphPad Prism software v. 5.00 (GraphPad Software, San Diego, USA),[34] the data were analyzed using the analysis of variance (ANOVA) test (one-way), followed by Tukey post-test, considering $p \leq 0.05$ as statistically significant.

Results and Discussion

Analysis by UHPLC-HRMS/MS

Through UHPLC-HRMS/MS, a scan was performed on the extracts in search of compounds with antioxidant potential, hence possibly justifying the potential of the extracts. In the first extract (G1) two compounds were identified: (epi)catechin (**1**) and procyanidin B-type (**2**) and in the G2 extract only procyanidin B-type (**2**) was identified. The fragmentation mass spectra of the compounds that were identified are available in Supplementary Information (SI) section and the chemical structures of substances identified by mass spectrometry are shown in Figure 1.

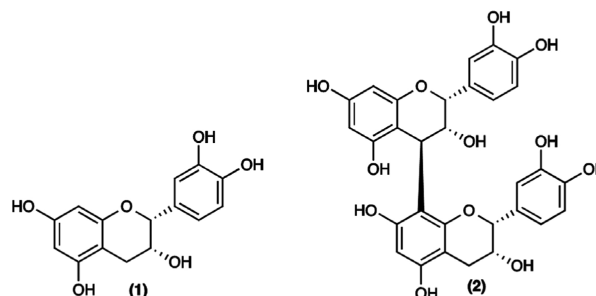

Figure 1. Chemical structures of substances identified by mass spectrometry.

Epicatechin (2*R*,3*R*)-configuration is a polyphenol. It is an enantiomer of a (+)-epicatechin and an antioxidant flavonoid, occurring especially in woody plants as both (+)-catechin and (–)-epicatechin (*cis*) forms.[33,35] Several studies, such as Dueñas *et al.*,[36] Leyva-Soto *et al.*,[37] Anitha *et al.*,[38] and Aree *et al.*,[39] show epicatechin as a potent antioxidant. Procyanidin B2 is a proanthocyanidin consisting of two molecules of (–)-epicatechin joined by a bond between positions 4 and 8' in a beta-configuration. It is a hydroxyflavan, a proanthocyanidin, a biflavonoid and a polyphenol. It derives from a (–)-epicatechin[33] and is presented in several studies as an antioxidant.[39-43]

In vitro antioxidant capacity

Table 1 shows the EC_{50} of G1, G2, and quercetin (QT). The use of more than one method has been shown to provide results that expressed with more accuracy the total antioxidant capacity, and DPPH$^•$, XOD, FRAP and ABTS$^{•+}$ assays are some of the most widely used methods for determining the *in vitro* antioxidant potential. The results of G1 and G2 were compared with values obtained for

Table 1. Antioxidant activity of extracts of *Guazuma ulmifolia* Lam. and QT

Sample	EC_{50}			
	DPPH$^•$ / (µg mL^{-1})	XOD / (µg mL^{-1})	FRAP / (mmol ET g^{-1})	ABTS$^{•+}$ / (mmol ET g^{-1})
Aqueous extract (G1)	11.46 ± 1.15^a	1.12 ± 0.21^a	2.41 ± 0.17^a	5.67 ± 0.85^a
Acetonic extract (G2)	8.45 ± 1.95^a	2.82 ± 0.26^b	2.83 ± 0.58^a	1.93 ± 0.56^b
Quercetin (QT)	3.08 ± 0.78^b	0.12 ± 0.0^c	6.15 ± 0.10^b	6.32 ± 0.75^a

EC_{50}: concentration that inhibited 50% of free radical. mol ET g^{-1}: millimolar equivalent of Trolox *per* gram of sample (for FRAP and ABTS$^{•+}$). Values ± standard deviation; n = 3. Different letters indicate significant difference ($p < 0.05$). QT: quercetin; DPPH: 2,2-diphenyl-1-picryl-hydrazyl; XOD: xanthine/luminol/xanthine oxidase; FRAP: ferric reducing antioxidant power; ABTS: 2,2'-azinobis(3-ethylbenzothiazoline- 6-sulfonic acid).

QT, a flavonoid with high antioxidant potential described in the literature.[44]

In DPPH$^{\bullet}$ tests, it can be seen that the extracts of *G. ulmifolia* Lam. have good antioxidant activity, capturing 50% of free radicals in the 11.46 ± 1.15 μg mL^{-1} range for G1 and 8.45 ± 1.95 μg mL^{-1} for G2. These potential antioxidant values are superior to other studies using plant extracts such as *Nectandra falcifolia* leaves (23.86 ± 0.16 μg mL^{-1}),[45] crude *Arrabidaea chica* extract (15.6 ± 1.82 μg mL^{-1}),[28] *Terminalia brasiliensis* Camb. (27.59 ± 0.82 μg mL^{-1}),[46] crude *Bauhinia ungulata* L. extract (21.87 ± 1.63 μg mL^{-1})[47] and *Garcinia brasiliensis* epicarp extract (47.46 μg mL^{-1}).[48] The values of G1 and G2 were not statistically different.

For xanthine/luminol/XOD tests, the EC_{50} was achieved in the concentration of 1.12 ± 0.21 μg mL^{-1} for G1 and 2.82 ± 0.26 μg mL^{-1} for G2. In that experiment, G1 was more effective than G2. Also, in this test, the drugs tested (G1 and G2) are more effective compared to other plant extracts such as *Calendula officinalis* extract (4.4 ± 0.9 μg mL^{-1}),[49] *Garcinia brasiliensis* epicarp extract (4.49 μg mL^{-1})[50] and *Pistacia lentiscus* (27.52 μg mL^{-1}).[51] These data indicate that the extracts obtained from *G. ulmifolia* Lam. have great potential antioxidant, justifying additional studies. According to Reynertson *et al.*,[52] samples less than 50 μg mL^{-1} that inhibit 50% of the radical are considered to be very active.

Through FRAP tests, the following results were obtained: 2.41 ± 0.17 mmol ET g^{-1} for G1 and 2.83 ± 0.58 mmol ET g^{-1} for G2. The values obtained are higher than those found by Fernandes *et al.*,[53] who studied fennel (0.04361 ± 0.00089 mmol ET g^{-1}), ginger (0.06436 ± 0.00279 mmol ET g^{-1}), mint (0.06794 ± 0.00075 mmol ET g^{-1}) and chamomile (0.15394 ± 0.0058 mmol ET g^{-1}). Comparing these values with other studies, it can be seen that G1 and G2 also have good antioxidant activity in the FRAP method.

The values obtained by the ABTS$^{\bullet+}$ method for G1 and G2 were 5.67 ± 0.85 and 1.93 ± 0.56 mmol ET g^{-1}, respectively, showing that both extracts have antioxidant capacity in this method as well, with G1 being more efficient than G2. In addition, the values obtained by G1 were very close to the values obtained by QT (6.32 ± 0.75 mmol ET g^{-1}), the value obtained by G1 being statistically equal to the value obtained by QT. This shows that G1, even though it is an extract, has antioxidant activity similar to quercetin, an isolated flavonoid, considered a potent antioxidant.

Cytotoxic effects of *Guazuma ulmifolia* Lam. on L-929 cells

To determine the optimum experimental conditions, cell viability was determined by the neutral red assay. L-929 fibroblasts were exposed to different concentrations of the G1 and G2 (125, 62.5, 31.25, 15.62, 7.81 and 3.90 μg mL^{-1}) for 24 h. The results are shown in Figures 2a and 2b.

Figures 2a and 2b show the cytotoxicity of G1 and G2 in L-929 cells. It can be observed that G1 and G2 were only cytotoxic in the concentrations of 125 and 62.5 μg mL^{-1}, killing 45 and 40%, for G1, and 51 and 52%, for G2, respectively. In the concentrations of 31.25, 15.62, 7.81 and 3.90 μg mL^{-1} there was no significant difference in relation to the negative control, which shows no cell death in these concentrations.

Several studies[26,27,54] consider that the drug is only cytotoxic when it kills more than 20% of viable cells, which happens only in concentrations of 125 and 62.5 μg mL^{-1} for G1 and G2, that is, both drugs are only cytotoxic at the highest concentrations. However, at concentrations in which G1 and G2 were active in capturing free radicals, there is no evidence of cytotoxicity with no significant difference from the negative control group. These results are supported by other studies using *Guazuma* extracts, such as Lopes *et al.*[21] and Felipe *et al.*[18]

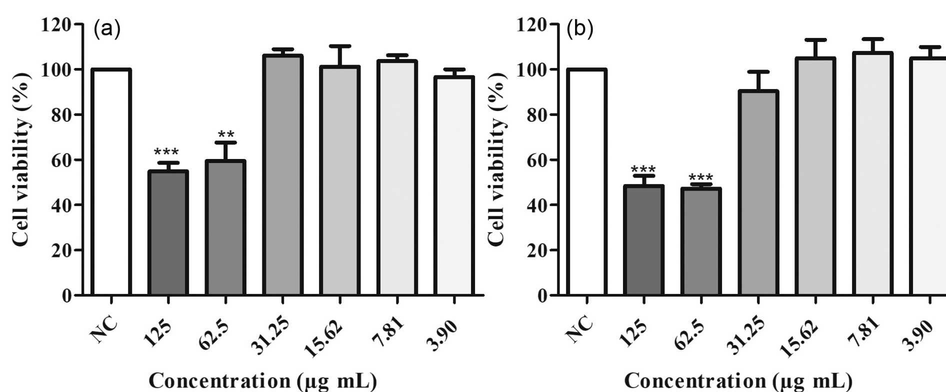

Figure 2. Cytotoxicity of G1(a) and G2 (b) in L-929 cells. L-929 cells (2.5 × 10^{5} mL^{-1}) were treated with different concentrations of the aqueous extract (a) and the acetonic extract (b) (125, 62.5, 31.25, 15.62, 7.81 and 3.90 μg mL^{-1}). Fibroblasts were treated and analyzed by neutral red assay (40 mg mL^{-1}). Readings were performed at 540 nm in a spectrophotometer. NC: negative control: untreated cells. The results are expressed as a percentage of control. $^{**}p < 0.01$, $^{***}p < 0.001$, significant difference compared to NC.

In vitro UV irradiation

In order to verify whether the drugs (G1 and G2) protect the cell from UV-A and UV-B irradiation, *in vitro* irradiation tests were performed. The results for the pre-treatment against UV-A irradiation with G1 and G2, are shown in Figures 3a and 3b, and against the UV-B irradiation with G1 and G2, are shown in Figures 4a and 4b, respectively.

It can be observed in Figure 3a that G1 was not able to protect the cells against UV-A irradiation, since the cell viability values (about 55%) were not statistically different from the UV-A control (wells without any drugs).

For G2 (Figure 3b), it can be seen that the concentration of 7.81 μg mL^{-1} (87.26% cell viability) was statistically different from the UV-A control (72.72% cell viability). That means that this concentration was able to protect the cells from UV-A irradiation, increasing cell viability by 19.99%. Comparing these data with other articles involving the use of extracts in photoprotection, such as that of Zaid *et al*.[55] who used pomegranate extract, it can be seen that they were able to protect cells using extract as pretreatment and increasing cell viability by 20%. However, they used a 5 times higher extract concentration (40 μg mL^{-1}) than what was used in the present study (7.81 μg mL^{-1}). The result obtained by G2 was much higher than that obtained by Daré *et al*.,[3] who used two extracts from *Senegalia polyphylla*; while the maximum value of reduction in cell death in this study was only 6%. G2 is also efficient when compared to the study of Silva *et al.*,[56] who used extracts obtained from *Nectandra hihua* leaves, where cell viability was restored by 13.52%. This shows how efficient G2 was when used as a pretreatment, that is, before irradiation, increasing cell viability and protecting cells against oxidative stress induced by radiation. Phenolic compounds have the presence of double bonds or aromatic

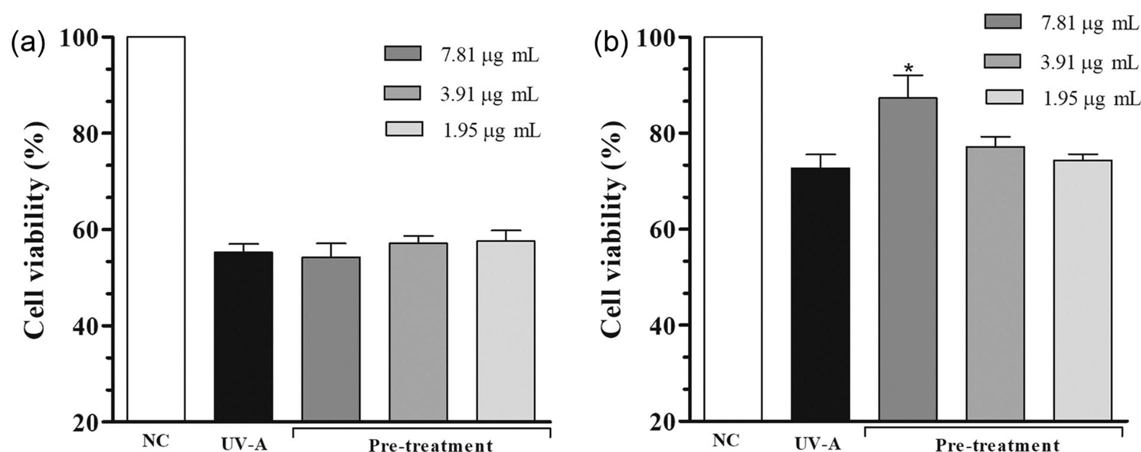

Figure 3. UV-A irradiation. L-929 cells (2.5 $\times 10^5$ mL^{-1}) were treated with G1 (a) and G2 (b) in different concentrations (7.81, 3.90 and 1.95 μg mL^{-1}). Fibroblasts were treated and analyzed by neutral red assay (40 mg mL^{-1}). Readings were performed at 540 nm in a spectrophotometer. NC: negative control: untreated cells and not irradiated. UV-A: untreated cells. The results are expressed as a percentage of control. *$p < 0.1$, significant difference compared to UV-A.

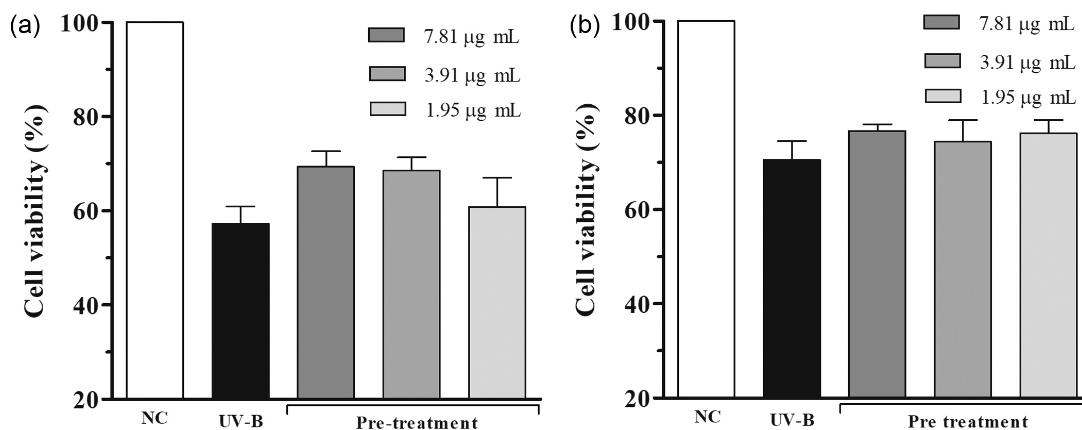

Figure 4. UV-B irradiation. L-929 cells (2.5 × 10^5 mL^{-1}) were treated with G1 and G2 in different concentrations (7.81, 3.90 and 1.95 μg mL^{-1}). Fibroblasts were treated and analyzed by neutral red assay (40 mg mL^{-1}). Readings were performed at 540 nm in a spectrophotometer. NC: negative control: untreated cells and not irradiated. UV-B: untreated cells. The results are expressed as a percentage of control.

rings in the molecular structure and for this reason, they are given UV absorption properties in the range of 200-400.[57] This and the antioxidant capacity of the compounds found in the extracts may have helped in the protective capacity against irradiation.

Regarding UV-B irradiation, Figure 4, there was no protection from extracts G1 and G2, since the values obtained were not statistically different from the UV-B control (wells without any drugs).

Scratch assay

Figures 5 and 6 presented the evaluation of the cell migration capacity via the scratch assay, using G1 and G2, respectively.

In Figures 5 and 7, it can be seen that only the concentration of 1.95 µg mL^{-1} of G1 in 24 h was different from the NC, showing a more expressive cell migration, being this 40%. The other concentrations do not seem to be different from the NC. On the other hand, in 48 h all the concentrations were statistically different from the control, being respectively 55.41, 59.04 and 65.16% for the concentrations 1.95, 3.90 and 7.81 µg mL^{-1}.

With G2 treatment, Figures 6 and 8, it can be seen that all concentrations after 24 h of treatment showed a greater cell migration related to the NC, being respectively 36.23; 39.79 and 37.89% for the concentrations 1.95, 3.90 and 7.81 µg mL^{-1}. In 48 h, only the concentration of 7.81 µg mL^{-1} of G1 was different from the NC, showing a growth of 75.20%.

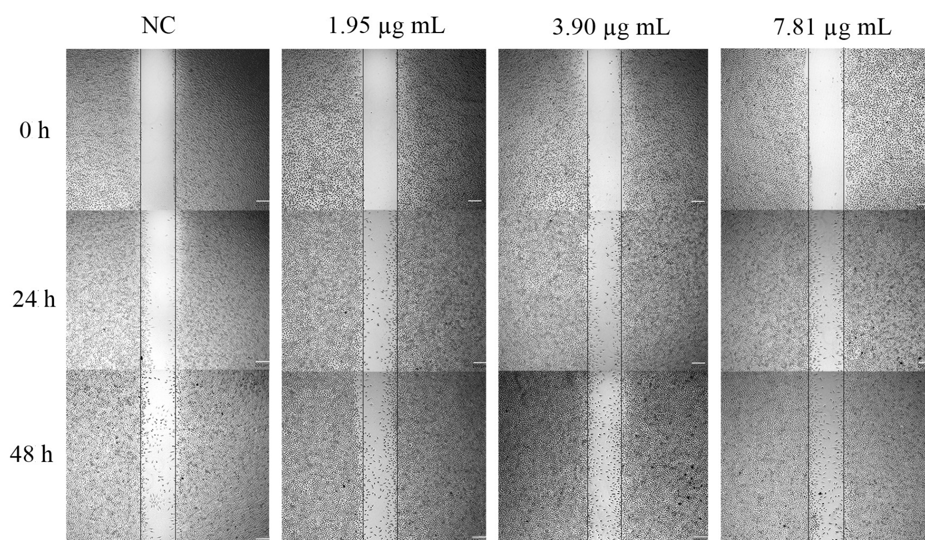

Figure 5. Evaluation of cell migration by the wound scratch assay in L-929 fibroblasts treated with G1 for 0, 24 and 48 h. Images were observed under an inverted microscope with phase contrast 5× magnification. NC: negative control; untreated cells.

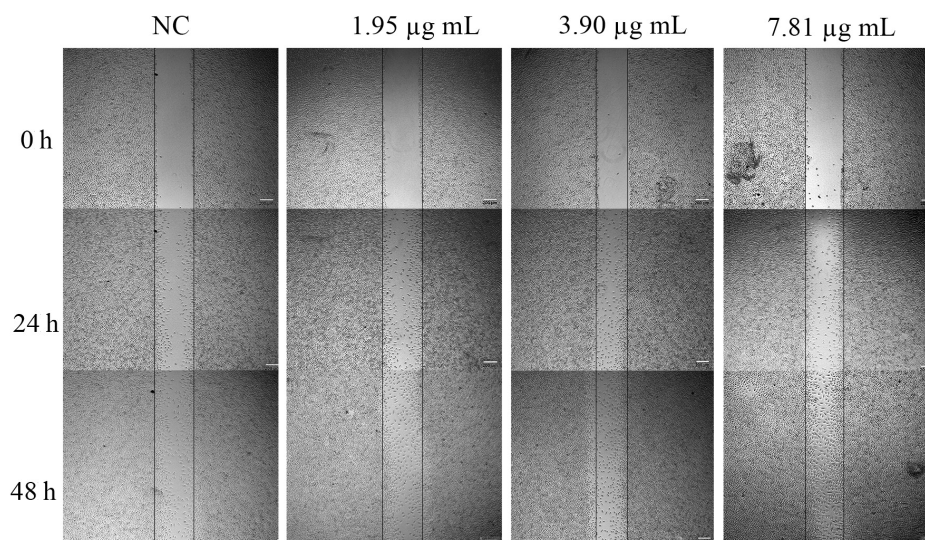

Figure 6. Evaluation of cell migration by the wound scratch assay in L-929 fibroblasts treated with G2 for 0, 24 and 48 h. Images were observed under an inverted microscope with phase contrast 5× magnification. NC: negative control.

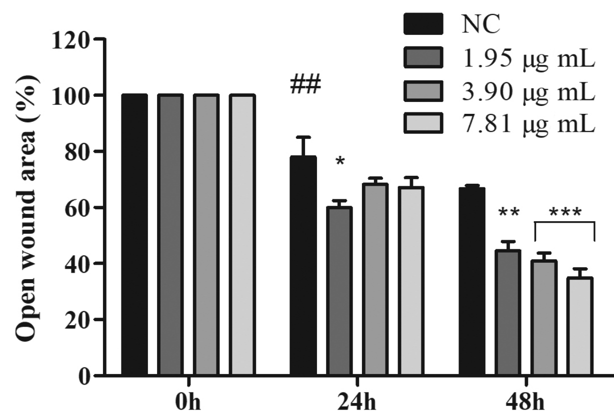

Figure 7. Evaluation of cell migration by the wound scratch assay in L-929 fibroblasts treated with G1 for 0, 24 and 48 h. Images obtained were quantified by ImageJ.[30] NC: negative control. $^{\#\#}p < 0.01$, a significant difference compared to NC of 0 h. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.1$, significant difference compared to 24 and 48 h NC.

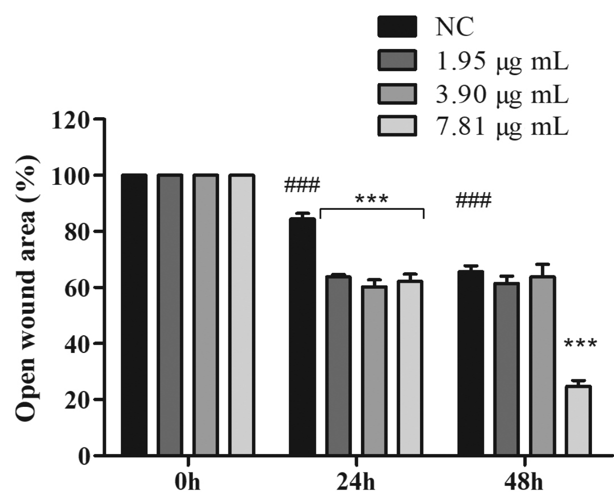

Figure 8. Evaluation of cell migration by the wound scratch assay in L-929 fibroblasts treated with G2 for 0, 24 and 48 h. Images obtained were quantified by ImageJ.[30] NC: negative control; untreated cells. $^{\#\#\#}p < 0.001$, a significant difference compared to NC of 0 h. $^{***}p < 0.001$, significant difference compared to 24 and 48 h NC.

Comparing these results with other studies, such as of Okur *et al*.[58] which tested *Phlomis rigida* Labill. extract and obtained a maximum healing rate of 50.1%, a lower rate was found in this study. Moreover, Silva *et al.*[56] who used an isolated compound with antioxidant potential called 2-acetylphenothiazine, obtained similar results in wound healing, showing 76.1% of cell migration in 48 h. These results show that *Guazuma* extracts have healing potential.

The healing activity can be attributed to antioxidant compounds, such as phenolic compounds. Plant extracts containing proanthocyanidins, flavonoids, polyphenolics, and polyphenols aid the healing process by first modulating superoxide anion and then increasing vascular endothelial growth factor expression, thereby increasing angiogenesis and blood flow.[58]

Endogenous antioxidant defenses: catalase, superoxide dismutase and glutathione reduced assays

Based on the results obtained in the photoprotection tests, it was chosen the G2 extract to perform the endogenous antioxidant defenses. Figure 9 shows the results of endogenous antioxidant defense assays: CAT, SOD and GSH assays.

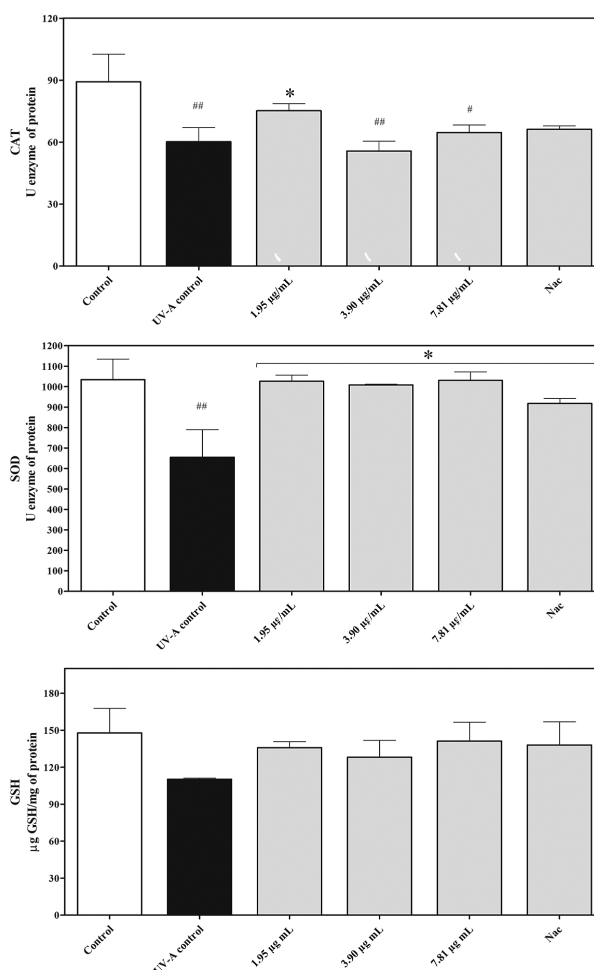

Figure 9. Evaluation of CAT, SOD and GSH enzymatic activity in UV-A-irradiated L-929 fibroblasts pretreated with G2. Control: non-irradiated and untreated cells. UV-A control: irradiated and untreated cells. Nac: *N*-acetylcysteine. $^{\#\#}p < 0.01$, a significant difference compared to control, $^{*}p < 0.1$, significant difference compared to UV-A control.

It can be observed that UV-A decreased the levels of CAT, SOD, and GSH enzymatic activity, reducing 40.90, 36.71, and 25.43%, respectively, compared with controls (non-irradiated and untreated cells). The pre-treatment with G2 at a concentration of 1.95 µg mL^{-1} significantly restored CAT enzymatic activity by 42.56%, compared with the UV-A controls (irradiated and untreated cells).

About SOD, the results show that all concentrations, 7.81, 3.90 and 1.95 µg mL^{-1}, significantly restored SOD

enzymatic activity by 57.31, 53.94 and 56.72% compared with the UV-A controls (irradiated and untreated cells). In the GSH assays, none of the concentrations were able to significantly restore GSH enzymatic activity.

Conclusions

The *G. ulmifolia* extracts proved to be efficient in capturing free radicals and wound healing, highlighting the G2 that protected effectively L-929 fibroblasts against UV-A-induced oxidative stress, in addition to restoring CAT and SOD enzymatic activity.

These results indicate the potential of the extract derived from *G. ulmifolia* as a source of natural actives for the development of topical products to protect the skin against oxidative stress, changes induced by sun exposure and wound healing. Besides, they provide a basis for further studies to evaluate the effectiveness of *Guazuma* extracts and their derivatives in preventing damage mediated by UV radiation and photoaging. For future studies, it would be interesting to isolate the compounds identified in the extracts and test them separately and *in vivo*.

Supplementary Information

The fragmentation mass spectra of the compounds that were identified are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Lennon A. de Araujo was responsible for conceptualization, methodology, software, verification, formal analysis, investigation, writing - original draft, visualization; Karine C. Nunes for conceptualization, methodology, writing-review and editing; João Carlos P. de Mello for resources; Celso V. Nakamura for resources, writing-review and editing, supervision; Raquel G. Gomes for writing-review and editing, supervision; Rosangela Bergamasco for resources, writing-review and editing, supervision, project administration, funding acquisition.

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