Mulateiro (*Calycophyllum spruceanum*) Stem Cell Extract: An Evaluation of Its Anti-Aging Effect on Human Adult Fibroblasts

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Abstract— In the present study, mulateiro (*Calycophyllum spruceanum*) stem cell (MSC) extract was evaluated for its antioxidant activity and anti-senescence effect against hydrogen peroxide (H_2O_2)-induced oxidative stress in human adult fibroblast (HAF). Mulateiro is used as a medicinal plant for the treatment of skin wounds, cuts, burns and is also known to help combat the skin aging effects, parasites and fungal infections. Cells were treated with MSC extract following the induction of H2O2 oxidative stress. The senescence associated β-galactosidase (SA-β-gal) activity was used to evaluate the Anti-aging effect. MSC extract demonstrated antioxidant activity and anti-senescence effect against oxidative damage in fibroblast cells and suppressed H_2O_2 stress-induced premature senescence in a concentration-dependent manner. At 0, 01 (0,1%) and 0,05 mg/mL (0,5%), MSC extract showed a positive effect by minimising cell cycle arrest and apoptosis induced by hydrogen peroxide. These findings provide scientific support for the potential use of mulateiro stem cell extract in treatment of skin disorders and as a skin anti-aging agent.

Index Terms— Mulateiro, Antioxidant, Hydrogen Peroxide, Fibroblasts, Plant Stem Cell, Extract, Antisenescence

1 Introduction

Individual genetic variation and external factors such as environmental conditions, nutrition, alcohol and diseases are part of a complex and inevitable biological process that is attributed to aging or senescence in the human body (De Magalhães, 2014). Aging and age-related diseases are commonly associated with increasing oxidative stress (Zong et al., 2015). Investigations have reported that plant-derived antioxidants are able to slow down replicative senescence and reduce stress-induced premature senescence (Jin et al., 2010).

Natural antioxidants are preferable for medical applications and food additives due to the reported carcinogenesis and hepatoxicity of synthetic antioxidants (Chunhabundit et al., 2012). Mulateiro (*Calycophyllum spruceanum*) belongs to the family of the *Rubiaceae.* It contains phenols with strong antioxidant properties that prevent cell aging by stopping the action and accumulation of free radicals. Traditionally, it is used as a tea bark, sheel powder and ointment resin for the preparation of shampoo, creams and moisturizers.

Mulateiro is well-known for its strong antioxidant activity which may explain its traditional use to stop the aging process of the skin (De vargas et al., 2016). In Brazil, it is commonly known as mulateiro and it is used as traditional medicinal plant to help fight the effects of aging, parasites and fungal infections in indigenous culture. Extracts from plant stem cells or dedifferentiated plant cells have been used in cosmetics (Schmid et al., 2008). Plant callus are similar to plant stem cell with the ability to differentiate, divide and give rise to cells that differentiate giving rise to new stem cells. Cultured callus-derived cells provide a cost-effective, environmentally friendly, and sustainable source of important natural products. The effect of MSC extract on aging and lifespan has not been studied.

In the present study, we investigated the effects of MSC extract on anti-aging activity using H_2O_2-stressed human adult fibroblast (HAF) cells. SA-β-gal activity was used as the biomarker indicating premature senescence of the cells. To date, there is no current report of the potential anti-ageing effects of mulateiro stem cell (MSC) extract, neither in vitro nor in vivo. This first study shows that MSC extract is absorbed by the cells in vitro and potentially enhance the viability and promote an antioxidant effect combating oxidative senescence.

2 Material and Methods

2.1 Plant Material

Mulateiro seeds were collected from plants growing wild in rural areas of Rio Branco-Acre, Brazil. Seeds were treated with ethanol 70% for 30 sec followed by 10% commercial bleach for 10 min and then rinsed twice with distilled water. Germination was achieved by placing the seeds in sterile vessels containing Murashige and Skoog (MS) medium supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} naphthalene acetic acid (NAA) and 1 mg L^{-1} 6- benzyl amino purine (BAP) and maintained in a growth chamber at 18-22°C with fluorescent light (16 hrs) and dark photoperiod (8 hr) until germination. Callus was produced from sterile cuts of the mulateiro plant, and maintained on a medium favouring continuous growth of the non- differentiated cells. Selected callus was chosen for suspension culture, harvested, washed with purified water and freeze-dried.

2.2 Preparation of MSC Extract

The freeze-dried MSC extract was ground to fine powder and extracted with 70% methanol (ethanol-water 70:30 vol/vol) using magnetic stirrer (250 rpm) overnight at room temperature. Extraction was repeated twice with 70% EtOH

the next day, using sonication for 10 min, and pooled. Ethanol was evaporated from the filtered extracts with Rotavapor, and the the dried extracts were stored at −20 °C before further analysis.

2.3 Cell Culture

Human adult fibroblast cells (HAF) were cultured in DMEM medium supplemented with 10% heat inactivated FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO_2. The medium was changed every 3–4 days. The cells were cultured to approximately 80% confluence, harvested with 0.25% trypsin-EDTA, and then subcultured for an additional 48 h in DMEM. For this study, the cells used were early passages from 4 to 10.

2.4 Cell Viability

Cell viability was measured using the MTT assay method, which relies on the ability of viable cells to metabolically reduce the tetrazolium salt MTT to a purple formazan product, which can be quantified colorimetrically. Fibroblast cells were seeded in a 96-well plate at a density of 1×10^4 cells/ml and cultured in DMEM for 24 h. Then, MSC extract at concentrations (0,05%, 0,1% and 0,5%) was added to cells. After being treated with the MSC extract concentrations for 24 h, 20 µl of MTT (1 mg/ml) was added to each well. The plates were incubated at 37°C for 4 hrs. Then the solution was removed and 100 µl of DMSO was added to each well. After keeping cells in the dark at room temperature for 24 h, absorbance values were determined at 570 nm using a microplate reader (ELx 800 Microplate Reader, Bio-TEK).

2.5 Antioxidant Activity by DPPH Assay

Antioxidative activity of MSC extract was measured according to the scavenging activity of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Brand-Williams et al. In short, 1ml of MSC extract at concentrations (0,05%, 0,1% and 0,5%) was mixed with 1ml of 0.1 mM DPPH solution in ethanol. The reaction was conducted in triplicate, shaken for 30 min and the decrease in absorbance was measured at 520nm using a microplate reader after 30 minutes in dark using UV-Vis spectrophotometer. At the end of the procedure, the ability to reduce DPPH in the controls and studied extract were expressed in terms of the amount of antioxidant required to reduce the initial concentration of DPPH by 50% (EC50) calculated from the linear regression equation of analytical curves constructed for each sample. Ascorbic acid was used as antioxidant standard. The inhibition % was calculated using the following formula.

Inhibition % = Ac-As/Ac×100

Where Ac is the absorbance of the control
As is the absorbance of the sample

2.6 Anti-Aging Effect of MSC Extract in HAF Cells

To investigate if MSC extract can recover human cells from exposure to oxidative stress, HAF cells were seeded at a density of 1×10^4 cells/well in a 96 well plate. After 24 h incubation, cells were stressed with 1 mM H_2O_2 for 1 h. Then cells were washed with 1X PBS and treated with 0,05, 0,1 and 0,5% of MSC extract for another 24 h. Cells without any treatment were used as healthy controls (HC). Cells with H_2O_2 treatment only were used as dead controls (DC). After 24 h treatment from stress, cells were used for the study of Senescence-Associated β-Galactosidase Activity.

Senescence-associated β-galactosidase (SA-β-gal) staining was performed as previously reported by Debacq-Chainiaux et al. Briefly, HAF cells were harvested with 0.25% trypsin-EDTA, washed in PBS, fixed with 3.7% paraformaldehyde for 15 min at room temperature, washed with PBS, and then incubated in freshly prepared SA-β-gal staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, pH 6.0) at 37 °C overnight.

At the end of the incubation, the solution was removed and the cell monolayers were washed twice for approximately 30 s with 0.5 mL of PBS at room temperature. Senescent cells were re-suspended in ice-cold FACS buffer and analysed immediately in a FACS Calibur flow cytometer (NSW, Australia). Propidium iodide was used as dead/living cell indicator for all the cell samples.

2.7 Statistical Analysis

All the experiments were performed in triplicates and data points indicate the standard deviation of three samples. Graphpad Software QuickCalcs Student's t-test was used for data evaluation and also for computing the significance level of $P < 0.05$ and $P < 0.01$.

3 Results and discussion

3.1 Cell Viability

Herein we report that *Calycophyllum spruceanum* (mulateiro), a traditional medicinal plant from the amazon basin, is well-known for its high antioxidant content that could prevent cell aging by stopping the action of so-called free radicals (Funasaki et al., 2016). To our knowledge, this is the first report on the application of mulateiro stem cell (MSC) extract on human adult fibroblast (HAF) cells. In the present study, the MSC extract concentrations tested demonstrated no cytotoxicity in HAF cells in vitro. Fibroblast cells held greater than 100% viability after 24 h incubation with stem cell extract from mulateiro (Figure 1). It is expected that concentrations of the stem cell extract lower than 0,005 mg/mL would also be non-toxic. With addition of 1mM H_2O_2 to the dead control (DC), cell

viability decreased up to 22%. On the other hand, the healthy control (HC) showed cell viability at 100%. Therefore, a concentration of 1 mM H_2O_2 was chosen for further experiments.

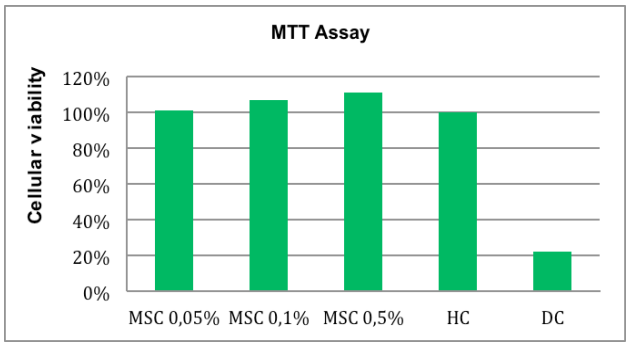

Fig. 1. Viability of fibroblast cells after treatment with 3 concentrations of MSC extract from 0,005 to 0,05 mg/mL and 1 mM H_2O_2 for 24 h. Data represent triplicate treatments and are expressed as mean ± SD (n = 3).

3.2 Evaluation of Antioxidant Activity

Some studies have shown some correlations between chemical and cellular measurements of antioxidant activity of mulateiro (De Vargas et al., 2016). The method of DPPH-radical scavenging activity is one of the most widely used method for screening the antioxidant activity of plant extract and was performed to evaluate the antioxidant activity of MSC extract. MSC extract slightly increased the DPPH radical scavenging activity in a dose-dependent manner (Figure 2). Several researchers have investigated the antioxidative activity of flavonoid compounds and have attempted to define the structural characteristics of flavonoids that contribute to their antioxidant activity (Chunhabundit et al., 2012).

The measurement of antioxidant activity is an important part of screening plant extracts that have the potential to protect cells from the damaging effects of oxygenated and nitrogenized free radicals, formed in oxidative processes. Previous studies have suggested that mulateiro extract exhibits potent free radical scavenging effects (De Vargas et al., 2016). MSC extract concentrations of 0,05%, 0,1% and 0,5% produced moderate to high DPPH scavenging activity. The highest DPPH scavenging activity was observed in concentration 0,5% (97 %) followed by AA (96%), 0,1% (92 %) and 0,05% (63.84%). From these results, we suggest that MSC extract has antioxidant activity.

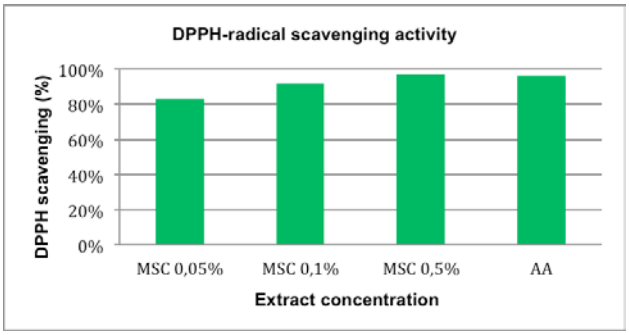

Fig. 2. DPPH radical scavenging activity of MSC extract. The amount of DPPH radicals was determined spectrophotometrically at 520 nm. Data represent the mean ± SE of three independent experiments. (AA) ascorbic acid.

3.3 Evaluation of Anti-Aging Effect

Aging is an inevitable process that is induced by external factors however can be slowed by down-regulating oxidative stress in the body, for instance, by supplementing with natural antioxidants. Natural antioxidants are of great importance for human health and have been reported to have anti-aging activity (Funasaki et al., 2016).

Cellular senescence was evaluated by counting the SA-β-gal-positive blue-stained senescent cells after H_2O_2 stress followed by MSC extract treatment. Cellular senescence caused by H_2O_2 oxidative stress in HAF cells has been widely used as a model to study cellular aging process (Frippiat et al., 2001). Stress-induced senescence of HAF cells caused by H_2O_2 was used to evaluate the anti-aging effects of MSC extract. It was observed that the ability of MSC extract could assist in the anti-senescence of HAF cells from the application of exogenous stress by H_2O_2.

Following 1 hour treatment with 1 mM H_2O_2 significantly ($p < 0.01$) increased SA-β-Gal positive cells by 52% in the dead control. MSC extract concentrations and ascorbic acid reduced the SA-β-Gal activity caused by H_2O_2, and showed a concentration-dependent effect (Figure 3). Concentrations 0,1% and 0,5% of MSC extract reduced SA-β-Gal activity by 11% and 12%, respectively. Accumulation of β-galactosidase occurred in H_2O_2 induced HAF cells and MSC extract exhibited protective effects with a significant decrease in β-galactosidase stained cells. The cells treated with ascorbic acid, used as standard also protected the cells from H_2O_2 induced cellular senescence.

All MSC extract concentrations showed significantly ($p < 0.05$) better recovery ability than the corresponding ascorbic acid treatment, although 352 µM ascorbic acid also significantly reduced the SA-β-Gal activity ($p < 0.01$) compared to H_2O_2 stress (Figure 3). This implied that MSC extract at these concentrations might have superior ability of protecting or recovering stress-induced premature senescence than ascorbic acid. These results suggest that MSC extract can be used as a potential anti-senescence treatment and has potent anti-aging activity in vitro.

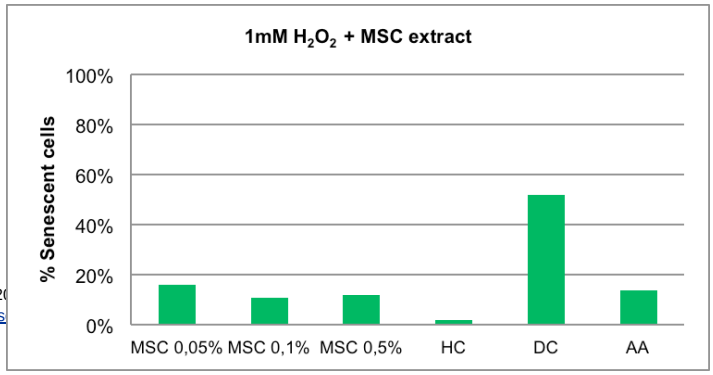

Fig. 3. Anti-senescence effect of MSC extract on H_2O_2 treated HAF cells. Cells were incubated for 1 h with 1 mM H_2O_2 as exogenous stress and then treated with 0,05%, 0,1% and 0,5% MSC extract for 24 h. (HC) Healthy cells - Blank; (DC) Dead cells - H_2O_2 only and (AA) ascorbic acid. Data are shown as percentage of SA-β-Gal positive cells (mean ± SE of three independent experiments).

In general, it is clear that MSC extract can protect human cells from stress-induced senescence. However, if this effect is long lasting or if frequent treatments are maintained, then this effect remains unknown and further studies should be conducted and further investigation is warranted. It is well known that plant antioxidants can protect the cells from premature aging caused by H_2O_2 (Varna et al., 2016). A previous report shows that a mixture of cocoa and green tea extracts, rich in polyphenols, and vitamin E significantly reduced UV stress-induced premature senescence from 51.67% to 38.81% in human dermal fibroblasts (Jorge et al., 2011). This suggested that MSC extract could not only assist cells to recover from acute H_2O_2 stress, but could also protect cells from chronic stress.

Further studies are needed to understand how MSC extract mediate the effects of the external stress factors on cells. For example, utilizing the comet assay to characterize the extent of DNA damage could help us understand the cellular components that are damaged by application of stress and would help to define which of the cellular processes are involved during recovery from stress (Sook et al., 2015). Further, the recovery ability of MSC extract against H_2O_2 HAF stress-induced premature senescence encourages the use of MSC extract as a potent anti-aging agent.

4 Acknowledgment

We thank all the authors for their contributions. Special acknowledges to the support from BIONORTE (Rede de Biodiversidade e Biotecnologia da Amazônia Legal), CAPES (Programa de Doutorado Sanduíche no Exterior) and CNPq (the Science without Borders Project).

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