Ilex Guayusa Leaf Extract as a protector of the scalp epidermal barrier and hair shaft against inflammatory process and oxidative stress induced by oxidative hair dye.

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ABSTRACT

The oxidative hair dye is the most expressive within this category and most contain the dye precursor p-phenylenediamine (PPD) and a coupling agent such as Resorcinol (RCN), which, after successive reactions under oxidative conditions, result in coloring of the hair. PPD is often used to contribute to the development of the final color, especially for dark shades. Exposure of the skin to this compound, in addition to irritation, can cause acute, subacute or chronic allergic contact dermatitis and induce the generation of reactive oxygen species (ROS), exacerbating the immune response and contributing to the impairment of the epidermal barrier. A good strategy to help prevent these symptoms is to associate an effective cosmetic ingredient with coloring to protect the scalp, avoiding or reducing the side effects of this inflammatory process [1,2]. The use of natural ingredients in cosmetics is growing every year, therefore, the association of antioxidant and anti-inflammatory properties of plants can be an interesting approach to mitigate allergic effects. *Ilex guayusa* leaf extract is an emblematic tree of the Amazon region, widely present in the region of Ecuador, Colombia, Peru and Bolivia with various uses in folk medicine; however, there are only few researches about the phytochemistry and biological activities in different applications such as nutraceutical or cosmetic [3]. The objective of this study was to explore the effectiveness of dry leaf extract of *Ilex Guayusa* as protective agent against inflammatory response and exacerbated oxidative stress, recovery of epidermal barrier and reduction of sensitivity in the scalp promoted by hair color. For this, the study was conducted in three steps: in vitro, ex vivo and clinic. In the first step, cells from hair follicle dermal papilla (HFDPCs) were incubated with 3 non-cytotoxic concentrations of the product concomitant exposure to chemical inducers (0.01 µM PPD and RCN + 3% H_2O_2) for further evaluation of radical protein formation and radical status factor (RSF), semi-quantitative assessment of mitochondrial super oxide using the fluorogenic probe MitoSOX ™ Red and quantification of the interleukin 8 (IL-8). In the second step, two *Ilex Guayusa* concentrations (0.5% and 1.0%) were prepared directly in the hair dye and evaluated in ex vivo scalp fragments culture and human hair shaft submitted to the dyeing process. In the third stage, a single-center, blind, comparative clinical study was conducted to prove the effectiveness of using 0.5% *Ilex Guayusa* in reducing scalp sensitivity induced by hair dye. Our results demonstrate that *Ilex Guayusa* can reduce the excessive synthesis of IL-8, an important inflammatory marker of the allergic contact dermatitis process, and recovers the epidermal barrier, strengthening the synthesis of filaggrin. In addition, reduces mitochondrial oxidative stress and protects the scalp and hair shaft from the action of free radicals, increasing hair strength and hydration effect. Clinically, we observed a reduction in scalp sensitization. These results together demonstrate that the use of *Ilex Guayusa* associated with hair coloring protects the scalp and hair shaft from damage caused by chemical inducers commonly present in hair colorings, improving consumer health and well-being.

MATERIALS AND METHODS

Standards, chemicals and solvents

Test-System: Human Follicle Papilla Dermal Cells (HFPDC) – (Cell Applications Inc.)

Positive Control: P-phenylenediamine dye (PPD) and Resorcinol dye (RCN) and Hydrogen peroxide (H_2O_2); A solution in the proportion of 10 µM PPD and RCN was prepared with 3% H_2O_2 in culture medium to perform the cytotoxicity test, as described in Methodology item 2, the results are shown in figure 1.

For the exposure of cultures as a positive control, a non-cytotoxic concentration of 0.01 µM PPD and RCN with 3% H_2O_2 was used.

Negative Control: Culture Medium: Human Follicle Papilla Dermal Cells (HFPDC) – (Cell Applications Inc.)

HFDPCs culture

HFDPCs were seed in culture bottles using the specific medium, supplemented with 1% gentamicin, and incubated at 37 °C, with 5% CO_2, until they reached confluence. These cells were used in the dose definition assay (Cytotoxicity), for this purpose they were seeded in 96-well plates at a density of 5x103 cells per well and a final volume of 200 µL. For the effectiveness assay, cells were cultured in 6-well plates at a density of 2.5x105, with a final volume of 3 mL. Collagen coating was carried out on all plates and culture bottles to allow cell adherence. At the end of the experiments, a Mycoplasma detection test was performed by luminescence, proving the ABSENCE of Mycoplasma in the cultures.

Test to define treatment concentrations – Cytotoxicity (data not shown)

To define the non-cytotoxic concentrations used in the treatment, the investigational product was solubilized in culture medium to assess the viability of cells exposed to concentrations of 100.00; 31.60; 10.00; 3.16; 1.00; 0.32; 0.01 and 0.03 mg/mL for 72 hours, using the MTT vital dye ((3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide). After the treatment, the optical density (OD) reading was performed at 570 nm. The same procedure was performed to define the non-cytotoxic dose of the chemical stress inducers (PPD + RCN + H_2O_2). Calculations to define the highest possible concentration that does not cause cell death were performed using the following equation for each concentration assessed:

% Viable Cells = DO mean treatment concentration * 100
DO Control group mean

Treatment protocol and chemical inducers

The cells were treated with concentrations of 0.32; 0.10 and 0.03 mg/mL, defined in the cytotoxicity assay, for 72 hours, concomitant with exposure to chemical inducers (PPD + RCN + H_2O_2) used as a positive stress control. At the end of this period, the cell lysate was collected for further quantification of the proposed mediator.

Quantification of mediator - RSF

The cell lysate was subjected to protein quantification using Bradford technique and then 10 µg/mL of protein from each sample was treated with 100 mM DMPO, 4.4 mM H_2O_2 and 50 µM $CuSO_4$, overnight at 37 °C. Subsequently, the formation of radicalized proteins in the samples was quantified by the immunoenzymatic assay technique (Elisa) using a commercially purchased kit, according to the manufacturer's instructions. RSF was determined according to the equation below:

RSF = Radical Proteins of stress group
Radical proteins of investigational product group

For RSF > 1 the product is protective against oxidative stress.

For RSF < 1 the product is a promoter of oxidative stress.

Quantification of mediator - MitoSOX™ Red

HFDPCs were incubated with 3 non-cytotoxic concentrations of the product for 72 hours concomitant exposure to chemical inducers (PPD + RCN + H_2O_2) for subsequent removal of treatments and an additional 18-hour incubation. Mitochondrial oxidative stress was semi-quantified by imaging the fluorescence emitted by the fluorogenic dye MitoSOX ™ Red. The cells were incubated with the fluorogenic dye MitoSOXTM Red at a concentration of 1 µM for 10 minutes at 37 °C. Then the MitoSOX™ Red was removed and DAPI was added to label the cells nucleus (1: 500) for 5 minutes. The cells were washed with PBS and photographed using the Leica DMi8 S automated microscope. Twelve images were collected per group and the analysis was performed using the ImageJ software.

Quantification of mediator – IL-8

The quantification of IL-8 in cell culture supernatants was performed by the immunoenzymatic assay technique (Elisa) using a commercially purchased kit, according to the manufacturer's instructions.

Clinic Study – Perceived Efficacy Assessment

A unicentric, blind, comparative clinical study was employed to assess the efficacy of the investigated product. Twenty female participants with a sensitive scalp were selected to participate in the study, which was followed-up by a dermatological evaluation and a questionnaire. A fixed amount of the investigated product was added to a commercial colouring cream and compared against the control group.

RESULTS AND DISCUSSIONS

Statistical Analysis

In the statistical evaluation, ANOVA non-parametric analysis of variance was used, followed by the Bonferroni post hoc test, allowing the evaluation of comparative results and data between the groups. The significance level of 5% was used (GraphPad Prism v6).

Mediator quantification - RSF

The results obtained showed that the exposure of cultures to chemical inducers promoted an increase in the formation of radical proteins of 54% ($P<0.001$), when compared to the baseline control group.

The investigational product reduces the formation of radical proteins by 27%; 25% and 24% ($P<0.001$) at concentrations of 0.32; 0.10 and 0.03 mg/mL, respectively, when compared to the group with exposure to chemical inducers, showing their ability to protect proteins from damage caused by free radicals. These results are shown in figure 1.

The RSF obtained was 1.37; 1.34 and 1.31 for concentrations of 0.32; 0.10 and 0.03 mg/mL, respectively, indicating that the product is a protector against oxidative stress in all concentrations evaluated.

Additionally, we can observe that the product has a radical protection factor of 76%, 72% and 68%, in concentrations of 0.32; 0.10 and 0.03 mg/mL, respectively.

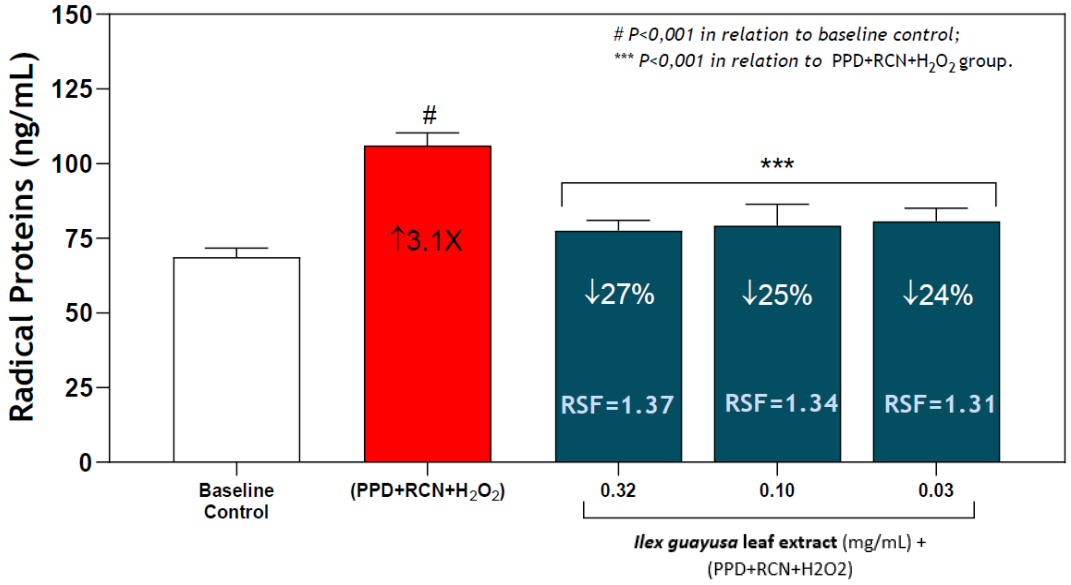

Figure 1. Quantification of radical proteins formation in HFDPCs culture treated with the investigational product *Ilex guayusa* leaf extract and exposed to chemical inducers. HFDPCs were treated for 72 hours with the investigational product and, at the same time, exposed to chemical inducers, for aftermost quantification of the radical proteins.

Mediator quantification - MitoSOXTM Red

In figure 2 we present a board with representative images of each experimental group, in red we can see the fluorescence emitted by the mitochondrial oxidative stress and in blue the cell nucleus is evident.

The results obtained showed that the exposure of cultures to chemical inducers promoted an increase in the mitochondrial oxidative stress of 73% (P <0.001), when compared to the baseline control group.

The investigational product reduces mitochondrial oxidative stress by 34%; 34% and 32% (P<0.001) at concentrations of 0.32; 0.10 and 0.03mg/mL, respectively, when compared to the group with exposure to chemical inducers, showing their ability to protect cells from damage caused by free radicals. These results are shown in figure 3.

Additionally, we can see that the product has the capacity to protect cells in relation to the maximum damage caused by chemical inducers of 81%, 81% and 76%, in concentrations of 0.32; 0.10 and 0.03mg/mL, respectively.

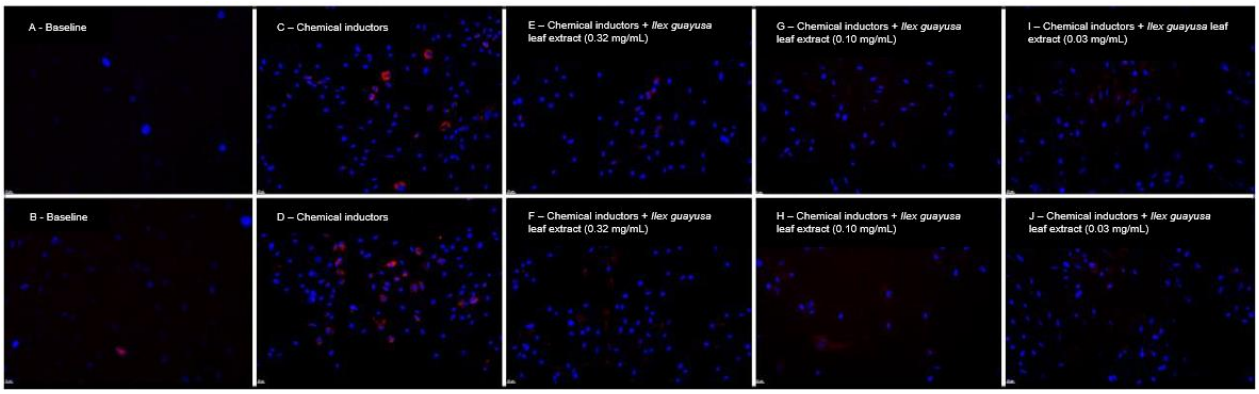

Figure 2. Imaging evaluation of mitochondrial oxidative stress in a culture of HFDPCs treated with the investigational product *Ilex guayusa* leaf extract and exposed to chemical inducers. HFDPCs were treated for 2 hours with the investigational product and, concomitantly, exposed to chemical inducers, for subsequent semi-quantification by image of the fluorescence emitted due to oxidative stress. A-B: baseline; C-D: Chemical inductors; E-F: 0.32 mg/mL; G-H: 0.10 mg/mL; I-J: 0.03 mg/mL. All images were obtained at 20X magnification.

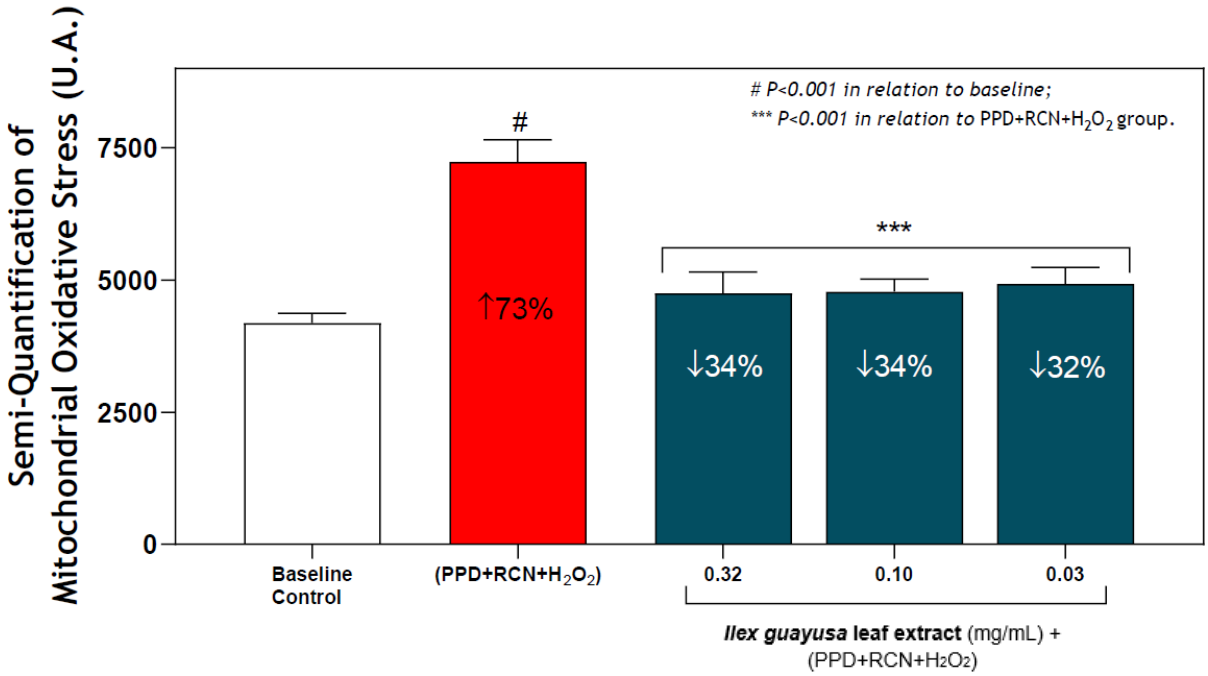

Figure 3. Semi-quantification of mitochondrial oxidative stress in HFDPCs culture treated with the investigational product *Ilex guayusa* leaf extract and exposed to chemical inducers. HFDPCs were treated for 2 hours with the investigational product and, concomitantly, exposed to chemical inducers, for subsequent semi-quantification by image of the fluorescence emitted due to oxidative stress.

Quantification of mediator – IL-8

The results obtained demonstrated that the exposure of cultures to chemical inducers promoted an increase of 3.1X ($P<0.001$) in the IL-8 synthesis, when compared to the baseline control group. The investigational product reduces the synthesis of IL-8 by 54% ($P<0.01$) and 33% ($P<0.05$), at concentrations of 0.32 and 0.10 mg/mL, respectively, when compared to the group with exposure to chemical inducers, showing its ability to protect cells from inflammatory stress. The concentration of 0.03 mg/mL had no significant effect in relation to the group with exposure to chemical inducers.

Additionally, we can observe that the product has a capacity of cell cultures protection against inflammatory stress caused by chemical inducers of 80% and 48%, in concentrations of 0.32 and 0.10mg/mL, respectively. These results are presented in Figure 4.

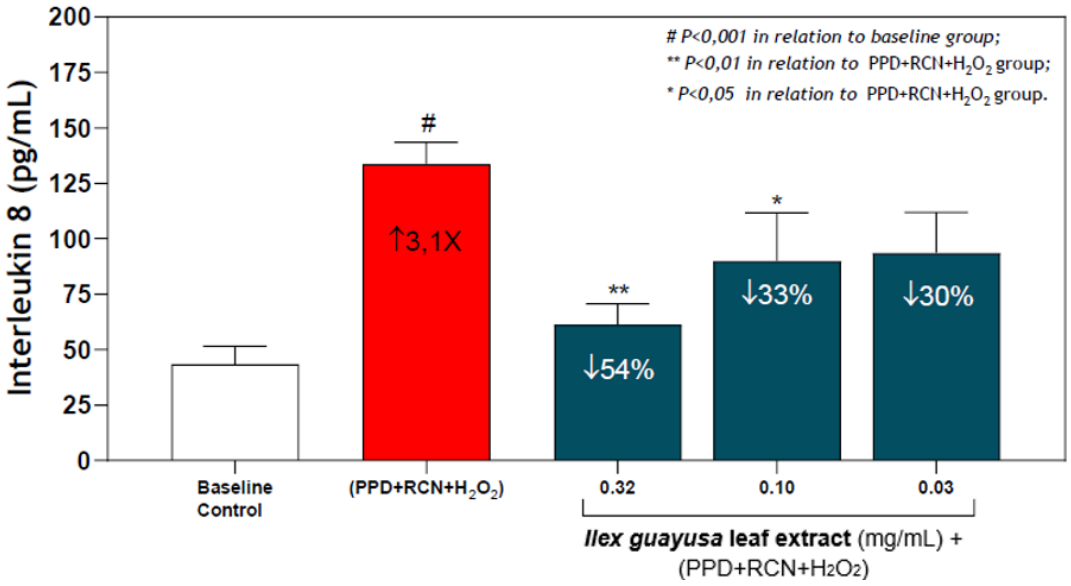

Figure 4. Quantification of interleukin 8 in HFDPCs culture treated with the investigational product *Ilex guayusa* leaf extract and exposed to chemical inducers. HFDPCs were treated for 72 hours with the investigational product and, at the same time, exposed to chemical inducers, for aftermost quantification of IL-8.

Perceived Efficacy Assessment

Clinically it was observed a reduction in scalp sensitization for 80% of the participants who responded the questionnaire claiming their scalp was “less sensitive than normal” after the usage of the investigated product, versus 56% from the control group.

CONCLUSIONS

Under the conditions in which the product described above was evaluated the data allow to conclude that: the investigational product has an antioxidant action reducing the damage to proteins caused by free radicals, with a radical protection factor of up to 76% and RSF of up to 1.37. All the presented data demonstrate that the product can prevent oxidative stress caused by chemical inducers commonly present in hair dyes and its efficacy was proved during clinical studies of simulation of real use.

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