

Evaluation of *in vitro* testing strategies for hazard assessment of the skin sensitization potential of “real-life” mixtures: the case of henna-based hair coloring products containing *p*-phenylenediamine

Renato I. de Ávila¹, Danilo F. M. C. Veloso², Gabriel C. Teixeira¹, Thaisângela L. Rodrigues¹, Tim Lindberg³, Malin Lindstedt³, Simone G. Fonseca⁴, Eliana M. Lima², Marize C. Valadares^{1,*}

¹Laboratory of Education and Research in *In Vitro* Toxicology – Tox In, Faculty of Pharmacy, Federal University of Goiás, Goiânia, GO, Brazil.

²Laboratory of Pharmaceutical Technology - FarmaTec, Faculty of Pharmacy, Federal University of Goiás, Goiânia, GO, Brazil.

³Department of Immunotechnology, Medicon Village, Lund University, Lund, Sweden.

⁴Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, GO, Brazil.

*Corresponding author. Address: Faculdade de Farmácia – Universidade Federal de Goiás, Rua 240 esquina com 5ª Avenida, s/n, Setor Universitário, Goiânia, GO, Brazil. CEP: 74605.170.

E-mail address: marizecv@ufg.br / marizeufg@gmail.com

Phone / fax: +55 62 3209-6039 ext. 202.

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Conflicts of interests

The authors declare no conflict of interest.

Abstract

Background. Allergic contact dermatitis reported to henna-based hair coloring products (HPs) has been associated with adulteration of henna with *p*-phenylenediamine (PPD).

Objectives. To develop a testing approach based on *in vitro* techniques that address key events within the skin sensitization adverse outcome pathway to evaluate allergenic potential of HPs.

Methods. The following *in vitro* assays were used to test the sensitizing capacity of hair dye ingredients: micro-direct peptide reactivity assay (mDPRA); HaCaT keratinocytes-associated IL-18 assay; U937 cell line activation test (USENS)/IL-8 levels; blood monocyte-derived dendritic cell test; genomic allergen rapid detection (GARD skin). Those techniques with better human concordance were selected to evaluate the allergenic potential of ten HPs.

Results. Contrasting to the label's information, chromatographic analyses identified PPD in all products. The main henna biomarker, lawsone, was not detected in one of 10 the products. Among the techniques evaluated by testing HDIs, mDPRA, IL-18 assay, GARD skin and U-SENS correlated better with human classification (concordances 91.7 to 100%) and were superior to the animal testing (concordance 78.5%). Thus, these assays were used to evaluate HPs, which were classified as skin sensitizers using different two-out-of-three approaches.

Conclusions. Our findings highlight toxicological consequences and risks associated of the undisclosed use of PPD in henna-based "natural" "real-life" products.

Keywords: allergic contact dermatitis, alternative methods, cosmetics, hair dyes, mixtures, skin sensitization.

1. Introduction

Hazard assessment of the skin sensitization potential of chemicals and/or “real-life” mixtures that may come into dermal contact, such as cosmetics, is mandatory in risk evaluation for classification and labeling for regulatory purposes (1-3). The objective is to identify potential health risks due to the use and handling of materials by consumers and workers, ensuring appropriate labeling of hazardous products and protection of public health (3, 4).

Contact allergy to chemicals is common, e.g. affecting 27% of the general population in Europe (5), the clinical manifestation being allergic contact dermatitis (ACD) (4, 6). The sequence of key events (KE) of skin sensitization induced by chemical contact allergens, such as skin protein reactivity and activation of keratinocyte and dendritic cells, has been summarized in an adverse outcome pathway (AOP) for skin sensitization (7). It represents the biochemical network that involves events spanning from the molecular initiating step to the *in vivo* outcome. Several *in chemico* and *in vitro* tests have been developed based on the skin sensitization AOP for toxicological analysis of materials without using animal models (e.g. the local lymph node assay, LLNA). Among these, six methods have been formally validated and adopted as OECD guidelines (8-10). However, guideline and non-guideline assays have all been developed focusing on pure substances only. Despite this, it has been proposed that some of them may be technically applicable to the evaluation of mixtures (8-10); in this sense, further studies are necessary. As it has been stated that a combination of *in vitro* methods can replace the conventional animal models, a testing approach based on *in vitro* techniques that address KEs within the skin sensitization AOP is also crucial to carry out the proper hazard evaluation of chemicals and finished products.

In the cosmetic field, severe allergic contact reactions have been associated with synthetic hair dye-based products by the occupationally exposed (e.g. hairdressers) and also by consumers who widely use these cosmetics on a regular basis (11, 12). In view of this widespread use, systematic evaluations have been conducted extensively regarding the safety profile of the use of synthetic ingredients in cosmetics. Consequently, several substances were banned or had their use restricted in cosmetics in the European Union, for instance (13).

Based on wash fastness and degree of permanence in the capillary fiber, synthetic hair dyes-based products available on the market are classified as bleaches (composed by hair lightening agents such as hydrogen peroxide), gradual (using metallic ingredients as salts of lead, bismuth or silver), temporary (water-soluble ingredients easily washed out in one shampoo rinse), semipermanent (which can withstand 6-8 times washing), and permanent dyeing (category that represent around 80% of all colorants that are hardly removable by shampooing, since to penetrate deeply into the hair shaft) (14, 15). These cosmetics may contain combinations of more than 100 different substances, e.g. dyes, couplers and fragrances; therefore they are complex mixtures of wide range synthetic compounds, many of them with potential skin sensitizing properties (12, 16, 17). Among these chemicals, the aromatic amine *p*-phenylenediamine (PPD) is a cost effective hair dye widely used, specifically in semipermanent and permanent products; however it is frequently associated with contact sensitivity reactions in humans after use of hair colorants (11, 17-20). Furthermore, PPD cross-reacts with chemically related molecules (20) eg used in cosmetics.

The prevalence of PPD allergy is reported to be high and differs between different regions (11). When added in hair colorants, PPD can provide black shades after reaction with secondary ingredients, such as resorcinol (18, 19). As other more efficient compounds have not successfully emerged, PPD is still considered a key hair dye precursor (15). It has generally been used as a marker for patch test screening to detect hair dye allergy (19, 21). Literature has also shown that PPD can be found in the plasma of exposed individuals,

demonstrating its capacity to easily penetrate the epidermal barrier in substantial amounts due to, in part, its low molecular weight (22, 23). PPD oxidation derivatives can be formed, such as Bandrowski's base (22, 23), an allergen which has been demonstrated to be 10-times more sensitizing than PPD in animal studies (24).

In view of this, botanical hair dye colorants like henna which can achieve a color change after a short period of time, have been considered as nontoxic alternatives to synthetic colorants (14), and henna cosmetics are in fact commonly used by pregnant women in Brazil. Corroborating its safe use, the Scientific Committee on Consumer Safety of the European Union recommended that henna is safe to use as a hair dye formulation, which commonly contains maximum of 20% henna powder suspended in 80% water (25).

Henna is a greenish powder obtained from the dried leaves of the *Lawsonia inermis* (Lythraceae) and the active dying phytochemical, the naphthoquinone lawsone (LAW, 2-hydroxy-1,4-naphthoquinone), can be found at 0.5-2% in the dried leaves (25). This natural material has traditionally been used by Islamic and Hindu population for different purposes, e.g. hair coloring, decorating the nails or temporary skin tattoos (26, 27). Nowadays, henna is extensively used worldwide due to the popularity of body art and hair dye products (28). Despite this extensive use, ACD to pure henna is extremely rare, and the allergic skin reactions reported have been associated with adulteration of henna with PPD (25, 26, 29-31), highlighting that these products deserve special attention by regulatory agencies.

Given the above, this study examined commercial henna products, recommended to reach different hair shades, for the presence of PPD and LAW by high performance liquid chromatography (HPLC). In addition, a battery of *in vitro* human-relevant assays addressing several KEs of the AOP for skin sensitization was used to test the sensitizing capacity of hair dye ingredients. After that, those techniques with better human concordance to hair dye ingredients previously tested here were selected and used to evaluate the allergenic potential of henna products.

2. Materials and Methods

2.1 Chemicals

Henna-based hair coloring cosmetic products were purchased between August and December 2016 at the local markets from Goiânia, GO, Brazil; the products were coded and the analyses conducted blindly (see Supplementary Table S1 for more details). Peptides containing lysine (Ac-RFAAKAA-COOH) and cysteine (Ac-RFAACAA-COOH) (both with a purity > 95%) were obtained from Genone Biotechnologies (Rio de Janeiro, RJ, Brazil). Ammonium hydroxide was purchased from Qhemis (Indaiatuba, SP, Brazil), whereas HPLC grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Avantor Performance Materials (Center Valley, Pennsylvania). Ammonium acetate, sodium phosphate monobasic and sodium phosphate dibasic were obtained from Synth (Diadema, SP, Brazil). Triton X-100 and basic red 51 were acquired from Amresco (Solon, Ohio) and Santa Cruz Biotechnology (Dallas, Texas), respectively. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and IL-4 used in peripheral blood monocyte-derived dendritic cell culture were purchased from PeproTech (Rocky Hill, New Jersey). Propidium iodide (PI), FITC-conjugated anti-human [CD14 (M5E2), CD86 (FUN-1), HLA-DR (L243), CD34 (581) and isotype control anti-IgG1 (MOPC-21)], PE-conjugated anti-human [CD54 (HA58), CD80 (L307) and isotype control anti-IgG1 (MOPC-21)] and PE Cy 7-conjugated anti-human CD11c (B-Iy6) antibodies were acquired from BD Biosciences (San Jose, California). FITC-conjugated anti-human CD1a (NA1/34) and PE-conjugated anti-human CD14 (TÜK4) antibodies were purchased from Dako (Santa Clara, California). Ficoll-Paque PLUS media, HyClone minimum essential medium alpha modification with L-glutamine, ribo- and deoxyribonucleosides (MEM- α) and TRizol reagent were purchased from Thermo Fisher Scientific

(Waltham, Massachusetts). Monocyte Isolation kit II, human (130-091-153) and rhGM-CSF (used in MUTZ-3 cell culture) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Direct-zol RNA MiniPrep column purification kit was acquired from Zymo Research (Irvine, California). Reagents used to conduct gene expression evaluation on NanoString GEN2 nCounter Analysis System, such as hybridization buffer, reporter codeset, capture probeset and nCounter cartridges, were obtained from NanoString Technologies (Seattle, Washington). Quantikine ELISA human IL-18 BPa kit or Human IL-18 Platinum ELISA were acquired from R&D Systems (Minneapolis, Minnesota) and eBioscience (Vienna, Austria), respectively. All other reagents were obtained from Sigma-Aldrich (St. Louis, Missouri).

2.2 Cell cultures

HaCaT human immortalized keratinocytes and U937 human histiocytic lymphoma cells were obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). HaCaT and U937 cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 medium, respectively, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), HEPES (4.5 mM), sodium bicarbonate (170 mM), penicillin (100 IU/mL) and streptomycin (100 mg/mL). MUTZ-3-derived cells (DSMZ, Braunschweig, Germany) were cultured in MEM- α supplemented with 20% (v/v) FBS and rhGM-CSF (40 ng/mL). All cell lines were maintained in a humidified atmosphere of 5% CO₂ in air at 37°C. To perform the assays, the cell viability was previously analyzed using TC20 (Hercules, California) or LUNA (Logos Biosystems, Annandale, Virginia) automated cell counters, according to manufacturer's instructions. A cell viability value > 90% was considered satisfactory to carry out the assays.

2.3 Identification of LAW and PPD

Solutions containing each product were prepared at 10 mg/mL in methanol, vortexed for 1 min and kept in an ultrasonic bath for 30 min at room temperature. After that, the solution was centrifuged at 14000 rpm for 5 min and the supernatant used for analysis. The presence and quantification of PPD and LAW levels in the samples was conducted by comparing their retention times with those of reference standards (10-1000 μ g/mL in methanol), using an Agilent 1260 series chromatographer (Palo Alto, California) equipped with a quaternary pump, online degasser, column heater, autosampler and diode array-detector (DAD). An injection volume for samples was 10 μ L onto the C18 column (4.6 x 250 mm, 5.0 μ m) (Agilent Technologies, Santa Clara, California). The temperatures of autosampler and column were 25 and 30°C, respectively. The scanning of UV spectrum was performed from 190 to 400 nm. Data acquisition was performed using Agilent OpenLab software (Palo Alto, California). The mobile phase consisted of a mixture of 0.1% (v/v) TFA in ultrapure water (A) and methanol (B), at 1.0 mL/min, with the following gradients: 1:1 over 2 min; 2:3 over 3 min; 3:7 over 2 min; 1:4 over 2 min; 1:9 over 1 min; and 9:1 over 2 min. The chromatograms were extracted at 252 nm.

2.4 Test materials and reference controls for skin sensitization evaluation

Ten hair dye ingredients were selected for testing using *in vitro* assays and evaluation of concordance with human data (see Table 5 for identities) to be posteriorly applied to conduct skin sensitization evaluation of henna products. Non-sensitizers [glycerol (GLY), dimethyl sulfoxide (DMSO) and sodium dodecyl sulfate (SDS)] and sensitizers [2,4-dinitrochlorobenzene (DNCB), eugenol (EUG) and 2-hydroxyethyl acrylate (2HA)] were used as reference controls. For *in chemico* assays, stock solutions (100 mM) of reference controls and hair dye ingredients were prepared in water, acetonitrile, DMSO or DMSO+acetonitrile; whereas solutions of henna-based hair coloring products (50 mg/mL) were prepared in methanol. In cell-based assays, these test materials solutions were dissolved in medium, deionized water or DMSO. The final DMSO concentration in the cell-based assays did not exceed 0.1% (v/v).

2.5 Micro-direct peptide reactivity assay (mDPRA)

A Varian ProStar chromatographer (Palo Alto, California) equipped with a low carry-over autosampler (model PS410), UV-visible detector (model PS325) and a quaternary pump (model PS240) was used to carry out the chromatographic analysis. An injection volume for samples was 10 μ L onto the C18 column (3.0 x 150 mm, 5.0 μ m) (Agilent Technologies, Santa Clara, California). The temperatures of autosampler and column were 25 and 30°C, respectively. Data acquisition was performed using Galaxie Chromatography Data System version 1.9.302.952 software (Palo Alto, California). The mobile phase consisted of 0.1% (v/v) TFA in ultrapure water (C) and 0.085% (v/v) TFA in acetonitrile (D), both at 0.35 mL/min. For the separation of peptides, a gradient of 10 to 25% (D) over 10 min followed by an increase to 90% over 3.5 min and a decrease to 10% (D) for 20 min of running. The chromatograms were extracted at 220 nm.

mDPRA, an analogous assay to the DPRA, was carried out according to a protocol previously described (32), which was based on OECD N° 442C (8) and DPRA DB-ALM protocol N° 154 (https://ecvam-dbalm.jrc.ec.europa.eu/methods-and-protocols/topic/sensitisation-and-allergy/key/t_41#). First, stock solutions of reagents used were prepared prior to use. Solutions (0.667 mM) of peptides containing cysteine and lysine were prepared in phosphate (100 mM sodium phosphate monobasic, 100 mM sodium phosphate dibasic; pH 7.5) and ammonium acetate (100 mM; pH adjusted to 10.2 by dropwise addition of ammonium hydroxide) buffers, respectively. Cysteine and lysine peptide solutions were then incubated with test materials at 1:15 (v/v) and 1:3 (v/v) ratios, respectively, in 0.25 mL glass inserts (Sun Sri, Rockwood, Tennessee) coupled into autosampler vials. The following volumes were added in each insert: 5 μ L test material, 20 μ L acetonitrile and 75 μ L cysteine peptide or 25 μ L test material and 75 μ L lysine peptide. The samples were incubated in the dark for 24 h at 25°C. After that, the samples were analyzed by chromatographer and peptide depletion (%) promoted by each test material was obtained according to the following equation: depletion (%) = $[1 - (\text{peptide peak area of test chemical} / \text{peptide peak area of control})] \times 100$. Negative depletion was considered as zero. Test materials with means of cysteine and lysine depletion < 6.38% were classified as non-sensitizers with no or minimal reactivity class. For sensitizers (mean of cysteine and lysine depletion > 6.38%), reactivity class was classified as following: low (6.38% < depletion $\leq$ 22.62%), moderate (22.62% < depletion $\leq$ 42.47%) or high (42.47% < depletion $\leq$ 100%).

2.6 HaCaT keratinocytes-associated IL-18 assay

Analysis of intracellular IL-18 production by HaCaT keratinocytes was conducted according to Corsini et al (33). In brief, cells (1.5×10^5 cells/mL) were seeded into 96-well plates and incubated overnight. After

that, they were exposed to eight different concentrations of test materials (0.2-1000 µg/mL), vehicle controls or culture medium only (control cells) for 24 h. The plates were then washed twice with PBS (100 µL/well) and cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay to determine the non-cytotoxic concentrations of the test materials that maintain cell viability $\geq 80\%$ (CV_{80}). HaCaT cells (1.5×10^5 cells/mL) were then seeded into a 24-well flat plate for IL-18 production evaluation. After overnight incubation, cells were exposed to culture medium only (control cells), vehicle controls (0.4% DMSO or medium) or non-cytotoxic concentrations (CV_{80}) of reference controls and test materials for 24 h. After that, cells were washed twice with PBS (1 mL/well) and cell lysates were obtained using 250 µL of PBS solution containing 0.5% (v/v) Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were stored at -80°C until analysis. IL-18 levels were quantified using the Quantikine® ELISA human IL-18 BPa kit (R&D Systems) or Human IL-18 Platinum ELISA (eBioscience) in accordance with the manufacturers' instructions, while the protein content of the cell lysates was measured by bicinchoninic acid protein assay kit (Sigma-Aldrich) using bovine serum albumin as standard. The IL-18 levels (pg) are expressed based on total intracellular protein content (mg) and the stimulation index (SI) was calculated as follows: $SI = \text{IL-18 in test material-treated cells} / \text{IL-18 in vehicle-treated cells}$. Materials with $SI \geq 1.2$ associated with a significant increase in IL-18 levels, when compared to vehicle treated cells, were classified as skin sensitizers.

2.7 U937 cell line activation test (U-SENS) and IL-8 levels analysis

CD86 expression in U937 cells after exposure to test materials was evaluated using U-SENS, according to OECD N° 442E (34). First, cytotoxicity of test materials was conducted to determine their CV_{70} (concentrations which promote viability $\geq 70\%$) in U937 cells. In brief, cells (5×10^5 cells/mL) were plated into 96-well plates with 100 µL/well. After that, 100 µL of five different concentrations of each test materials (0.4-200 µg/mL) were added and the plates were incubated for 45 h. The cells were then washed twice with ice cold-PBS and cell viability was determined by propidium iodide (PI) (3 µg/mL) staining. To evaluate CD86 expression, U937 cells were exposed to five different concentrations $< CV_{70}$ or ≤ 200 µg/mL (the highest concentration allowed) of test materials. After 45 h of incubation, cells were washed twice with 200 µL of ice-cold buffer (PBS + 5% FBS, v/v) at 1000 rpm, at 4°C for 5 min. After that, they were incubated with staining solution containing buffer (95 µL) and FITC-conjugated anti-human CD86 monoclonal antibody (5 µL) or FITC-isotype control IgG1 (5 µL). After 30 min of incubation at 4°C , cells were washed once with ice-cold buffer and PBS followed by addition of 100 µL of PI (3 µg/mL) solution for analysis using flow cytometer (BD FACSCanto II, BD Biosciences, San Jose, California) recording 10000 events. Dead cells (PI+) were excluded from the CD86 expression analysis. Data were calculated as stimulation index (SI) according to the following equation: $SI = [(\%CD86_{\text{treated}} - \% \text{ isotype IgG1}_{\text{treated}}) / (\%CD86_{\text{control}} - \% \text{ isotype IgG1}_{\text{control}})] \times 100$. A positive response for skin sensitization was considered when $SI \geq 150\%$. EC_{150} , concentration at which the test material induced a SI of 150%, was also obtained through a linear regression.

To quantify IL-8 levels, U937 cells were exposed to EC_{150} or 200 µg/mL of each test material. After the incubation period, the cell supernatants and lysates were stored at -80°C to await IL-8 analysis using BD OptEIA™ human IL-8 ELISA set kit (BD Biosciences), in accordance with the manufacturer's instructions. Extracellular IL-18 levels are expressed in pg/mL, while intracellular IL-18 content are expressed in pg/mg of total intracellular protein content, which was measured by bicinchoninic acid protein assay kit (Sigma-Aldrich) using bovine serum albumin as standard. A test material was considered as sensitizer according to

the same prediction model described in HaCaT keratinocytes-associated IL-18 assay, i.e. $SI \geq 1.2$ plus significant increase in IL-18 levels in comparison to vehicle treated cells.

2.8 Peripheral blood monocyte-derived dendritic cell (PBMDc) test

This assay was conducted based on the protocol described by Reuter et al (35). In a first step, PBMDcs exposed to test materials were stained with PI (3 $\mu\text{g/mL}$) and analyzed by flow cytometer to determine CV_{80} value (test material concentration which promote viability $\geq 80\%$). After that, CD86 expression was analyzed after exposure to five different concentrations $< CV_{80}$ or $\leq 200 \mu\text{g/mL}$ of test materials. For the assays, blood samples were collected from healthy male donors, after approval of the Ethics Commission of Federal University of Goiás (protocol UFG nº 2.324.753/2017) in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

In brief, human whole blood was used to obtain mononuclear cells by Ficoll-Paque PLUS density gradient separation. Monocytes were then isolated by negative depletion using the Monocyte Isolation kit II, human (Miltenyi Biotec), according to the manufacturer's instruction. An aliquot of monocytes-containing suspension was incubated with anti-CD14 antibody (10 μL) in PBS (90 μL) for 30 min followed by washing in PBS. CD14 phenotype was then measured by flow cytometer. Monocytes-containing samples from each donor with CD14⁺ cells $> 70\%$ were used to obtain PBMDcs. Cell suspension (1×10^6 cells/mL) in culture medium were then plated into 6-well plates with 2 mL/well. The culture medium used was RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, HEPES (4.5 mM), sodium bicarbonate (170 mM), penicillin (100 IU/mL), streptomycin (100 mg/mL), rhGM-CSF (25 ng/mL) and IL-4 (25 ng/mL). After 5 days of incubation, aliquots of samples of each donor were incubated with FITC-anti-CD14 (10 μL), PE Cy 7-anti-CD11c (0.75 μL) and FITC-anti-CD86 (10 μL) antibodies in PBS (final volume: 100 μL) for 30 min. After that, cells were washed with PBS, resuspended with 200 μL PBS containing PI (3 $\mu\text{g/mL}$) and analyzed by flow cytometer. Debris, lymphocytes and dead cells (PI⁺) were excluded from the analysis. PBMDcs samples, with CD86⁺ cells $< 20\%$ and CD11c⁺ cells $> 80\%$, were then plated into 24-well plates and exposed to test materials for cytotoxicity or CD86 expression assessments. Analysis of CD86 expression after treatment was calculated as follows: $\Delta\text{CD86} = (\% \text{CD86}_{\text{treated}} - \% \text{isotype IgG1}_{\text{treated}}) - (\% \text{CD86}_{\text{control}} - \% \text{isotype IgG1}_{\text{control}})$. Test material was considered as skin sensitizer when $\Delta\text{CD86} > 20\%$ for any concentration tested. EC_{20} , concentration at which the test material induces a $\Delta\text{CD86} = 20\%$, was also obtained through a linear regression. Each test material was tested in at least five independent assays, corresponding to five different donors.

2.9 Genomic allergen rapid detection (GARD skin)

Measurements of transcriptional levels of 200 genomic biomarkers, referred as the GARD skin prediction signature, were conducted using NanoString nCounter digital bar-coding platform, according to the protocols previously published (36, 37). In brief, experiments were performed using three independent batches of cells. Flow cytometric phenotypic analysis was performed for each cell batch using the following antibodies: FITC-conjugated anti-human CD1a, CD34, CD86 or HLA-DR, and PE-conjugated anti-human CD14, CD54 or CD80. FITC- or PE-anti-IgG1 were used as isotype controls, while staining with PI (1 $\mu\text{g/mL}$) was performed for cell viability analysis. As a first step, RV_{90} values (test material concentration inducing 90% relative viability, when compared to unexposed cells) were determined by cytotoxicity analysis. For that, cells (2×10^5 cells/mL) were seeded in 24-well plates and exposed to different concentrations of test materials, vehicle controls (deionized water or DMSO 0.1%) or culture medium only (control cells) for 24 h.

Afterwards, cells were washed twice with PBS-BSA (1%, w/v) and stained with PI (1 µg/mL) for flow cytometry analysis to assess cell viability. When RV_{90} for each material was determined, cells (2×10^5 cells/mL) were seeded in 12-well plates and exposed to each test material for 24 h. Thereafter, cells were lysed in TRizol reagent and stored at -20°C until total RNA extraction, which was determined using Direct-zol RNA MiniPrep column purification kit (Zymo Research, Irvine, California), according to manufacturer's instructions. The integrity and concentration of total RNA in each sample were assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara), following the protocol provided by the supplier. For gene expression analysis on NanoString GEN2 nCounter Analysis System (NanoString Technologies), RNA samples were thawed on ice and set to a concentration of 20 ng/µL. After that, 5 µL of RNA solution of each sample were mixed with 8 µL master mix (hybridization buffer + Reporter CodeSet) and 2 µL Capture ProbeSet, followed by hybridization process through incubation in a ³Prime thermal cycler (Bibby Scientific, Staffordshire, UK) at 65°C for 24 h. Afterwards, samples were transferred to the GEN2 Prep Station 5s for post-hybridization processing using the high sensitivity protocol. nCounter cartridges were then sealed with an adhesive tape and transferred to the Digital Analyzer 5s for data collection using maximal data resolution (555 fields of view). After conclusion of this step, the Reporter Code Count files (RCCs) were downloaded from the machine and analyzed using the SenzaGen software version 1.0 (SenzaGen, Lund, Sweden), which conducted the Nanostring quality control as well as data normalization. A test material was classified as a skin sensitizer when the support vector machine (SVM) median output value of the three independent replicates were > 0.

2.10 Statistical Analysis

Data are expressed as mean or mean ± SD of at least three independent assays. Statistical analysis was performed using Graph Pad Prism 5 software version 5.0 for Windows 98 (San Diego, California). The inter group variation was measured by one-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test, respectively. Statistical significance was established as $P < 0.05$.

3. Results

3.1 Establishment of testing approaches for skin sensitization evaluation

To establish a testing approach with good human concordance for skin sensitization hazard assessment of cosmetics, such as henna products, ten hair dye ingredients were previously selected. These chemicals were tested in different *in vitro* guideline and non-guideline platforms, including protein binding (mDPRA); keratinocyte activation (HaCaT keratinocytes-associated IL-18 assay); and dendritic cell activation (U-SENS and IL-8 measurements, PBDC test, and GARD skin). In parallel, reference controls were tested, including non-sensitizers (GLY or SDS) and sensitizers (DNFB, EUG or 2HA). DMSO at 0.1% (v/v), considered as non-allergen, was also tested as vehicle control, to solubilize some chemicals, in the cell-based assays.

The mDPRA data are shown in Table 2. With the exception of SDS, a well-known false positive in LLNA, this assay was able to correctly identify the skin sensitization potential of the reference controls. All hair dye ingredients were predicted as sensitizers with reactivity classes from moderate to high. In addition, it was verified a higher peptide depletion for cysteine than lysine.

Fig. 1 shows the results obtained using the HaCaT keratinocytes-associated IL-18 assay, which classifies a material at non-cytotoxic concentration (CV_{80}) as skin sensitizer when it triggers a $SI \geq 1.2$ associated with a significant increase in IL-18 levels. The reference controls used (DMSO, SDS, GLY, EUG

and DNCB) were correctly identified as negative or positive for skin sensitization hazard. Among the 10 hair dye ingredients, LAW and 5OC did not trigger increase of the IL-18 levels, thus they were classified as non-skin sensitizers. PPD (811.49 pg/mg), DO3 (728.27 pg/mg), 2A3 (561.56 pg/mg), 14D (521.0 pg/mg) and BR51 (462.66 pg/mg) promoted the highest IL-18 levels when compared to the non-exposed cells (93.82 pg/mg).

In relation to U-SENS data (Table 3), U937 cells were exposed to non-cytotoxic concentrations of test materials for 45 h for evaluation of CD86 expression. Except for the irritant SDS, reference controls tested were correctly identified regarding to their potential to trigger skin sensitization (DNCB and EUG) or not (GLY and DMSO). In addition, all hair dye ingredients were classified as skin sensitizers. In parallel to U-SENS, intra and extracellular IL-8 levels in U937 cells were also quantified. In both analyses, reference controls were correctly predicted, now including SDS (Fig. 2A and B). Regarding hair dye compounds, only three (14D, RSC and DO3) did not trigger significant increase of intracellular IL-8 levels (Fig. 2A), whereas four substances (PPD, 5OC, DO3 and BR51) were negatives in the extracellular IL-8 levels analysis (Fig. 2B).

Moreover, PBMD test was performed to conduct the flow cytometric measurement of CD86 expression in a primary human cell model. As show in Table 4, pro-hapten EUG was the only reference substance wrongly predicted as non-allergen, which suggest that the cell system used in this assay fails to promote its enzyme-mediated activation. Also, pro-hapten hair dye RES was not correctly identified as skin sensitizer. In addition, HQ was the only hair dye ingredient able to trigger increase of CD86 expression, therefore classified as allergen.

Transcriptional quantitative analysis of 200 genes, referred as the GARD skin prediction signature, was also conducted using a myeloid cell line as a surrogate for dendritic cells. The analyses were performed in triplicate using different cell batches. SVM decision values were assigned to each sample replicate and those mean values of triplicates > 0 classified the test materials as skin sensitizers. Previous published data have shown that the reference controls GLY, SDS, DNCB and EUG are correctly predicted regarding the skin sensitization hazard by GARD skin (36-38). In the same way, here we also showed that this assay properly identified DMSO and 2HA as skin non-allergen and allergen, respectively (Fig. 3 and Supplementary Table S2). Moreover, all hair dyes tested triggered modulation of the genes involved in the skin sensitization prediction of GARD skin, being classified as skin sensitizers (Fig. 3 and Supplementary Table S2).

Table 5 summarizes the predictions of the seven *in vitro* assays evaluated for skin sensitization hazard of hair dye ingredients as well as the reference controls. In comparison to animal data, mDPRA, U-SENS and GARD skin (76.9-78.6%) showed similar concordance, whereas the other techniques showed inferior values (50-64.3%). When compared to human classification, intra and extracellular IL-8 measurements and PBDC test results also showed inferior concordance of 69.2%, 76.9% and 38.5%, respectively. On the other hand, these values for mDPRA, IL-18 assay, U-SENS and GARD skin were superior in comparison to the human repeated insult patch test, showing that their *in vitro* predictions correlate better with the human classification, in addition to being superior to the animal testing (concordance 78.5% vs. human data). Among them, GARD skin reached the best prediction (concordance 100%), whereas mDPRA (concordance 91.7%), IL-18 assay and U-SENS (both concordance 92.3%) showed similar predictive capacity. Therefore, these four promising techniques were selected to establish an *in vitro* integrated testing strategy (ITS) approach to evaluate the skin sensitization potential of henna-based hair coloring products.

3.2 Label and HPLC analyses

As a first step of the evaluation, ten henna-based cosmetics for different hair shades, marketed in Brazil, were characterized as to their ingredients declared by the manufactures. Therefore, the labels were analyzed and chromatographic analyses were performed to check the presence or absence of PPD and the henna biomarker LAW. The results are summarized in Table 1 and Supplementary Table S1.

Among the ten products analyzed, seven were for hair use (products no. 1-7) and three for eyebrow application (products no. 8-10). Eight of them were produced in Brazil, one in France (product no. 7) and another one in India (product no. 10). In the label analysis, only two products (no. 2 and 8) declared the presence of PPD. In addition, in these products the presence of other botanical ingredients (products no. 2-10) and synthetic substances (products no. 2, 3, 4, 7 and 8), including skin sensitizers such as resorcinol, HC Red no. 3, *m*-aminophenol and *p*-aminophenol was mentioned. Although the quantities of the declared ingredients were not provided on the labels, their order was arranged in descending order of amount as stated by Brazilian legislation, i.e. the first ingredient will be the one with the highest concentration, while the last ingredient listed will be the one with the lowest content in the final product.

Since all products analyzed were declared as henna cosmetics by the manufactures, the presence of LAW, the main active phytochemical of henna, was expected in all samples. However, HPLC analysis showed no LAW level in the product no. 2, suggesting falsification. Product n° 1 showed the highest levels of LAW (0.518%), whereas the other products showed mean values of 0.036% (products no. 5 and 10), 0.140% (products no. 3, 6, 8 and 9) and 0.340% (products no. 4 and 7). Furthermore, the presence of PPD was declared on the products no. 2 and 8 only by the manufactures. However, this substance was detected in all products, suggesting undisclosed adulteration of the products no. 1, 3-7, 9 and 10. The highest levels of PPD were found in the products no. 2 (2.97%), no. 5 (4.32%), no. 8 (2.54%) and no. 10 (3.35%).

The representative chromatograms of the HPLC analyses are shown in supplementary Fig. S1. The HPLC method developed showed retention times of 2.3 and 8.1 min for PPD and LAW, respectively. The calibration curves for quantification of both substances showed adequate linear tendency. The responses were linear throughout the calibration range with the coefficient (*r*) greater than 0.99. The absolute quantification limits were 5 and 500 µg/mL for both PPD and LAW.

3.3 Evaluation of henna products in the testing approach established

Ten commercial henna-containing hair coloring cosmetics were evaluated using different *in vitro* techniques addressing the KEs of skin sensitization AOP, previously established by testing hair dye ingredients. In general, all endpoints evaluated were increased in henna products when compared to hair dye ingredients tested alone. Thus, it is suggested that the presence of PPD as well as other sensitizing chemical may promote associative effects culminating on the increase of the skin sensitization potential of hennas, changing therefore, their toxicological profile.

Peptide reactivity results using mDPRA are shown in Table 2. In general, all products triggered higher mean peptide depletion (63.28%) than that one found for hair dye ingredients tested alone (51.75%). In relation to the type of peptide, depletion values of cysteine were greater than that of lysine. As a consequence of the high peptide depletion, the products were classified as skin sensitizers with high reactivity class.

Moreover, intracellular IL-18 levels in HaCaT cells exposed to the henna cosmetics were significantly increased for eight products (no. 1-6, 9 and 10) ($P < 0.05$) associated with SI values > 1.2 (Figure 1A); thus they were classified as positives for this endpoint. Interesting, henna products triggered an IL-18 level mean value of 480.47 pg/mg, which was higher than that one observed for the pure substances tested alone (340.17

pg/mg). The highest IL-18 levels were found for the products no. 2 (543.68 pg/mg), no. 3 (543.77 pg/mg), no. 5 (600.48 pg/mg) and no. 9 (691.43 pg/mg) (Figure 1A).

In relation to the U-SENS results (Table 3), henna products no. 3 and 4 did not promote activation of the U937 cells as noted by the analysis of the increase of CD86 expression. On the other hand, GARD skin data showed that all products tested triggered modulation of the 200 genes included on the GARD skin prediction signature, since their SVM mean decision values > 0 (Fig. 3 and Supplementary Table S2). In general, as observed in the other tests, SVM values were increased in henna products (mean = 4.76), when compared to those obtained for hair dye ingredients tested alone (mean = 2.75).

Table 5 summarizes the *in vitro* predictions of single assays as well as when combined in two-out-of-three strategies, i.e. a material testing positive or negative in any two of the three platforms is classified as a sensitizer or a non-sensitizer, respectively. It is interesting to note that mDPRA or GARD skin classified the henna products as skin sensitizers in the same way as the combination of techniques involving different endpoints.

4. Discussion

Aiming to evaluate the contact allergy potential of ten commercial henna-based hair dye products, containing different levels of PPD, a human-relevant testing approach was developed based on the current demand for animal-free testing for cosmetic safety assessment. The focus was on using techniques that address KEs within the skin sensitization AOP. Among the techniques investigated, mDPRA, IL-18 assay, U-SENS and GARD skin showed the best human correlation for the hair dye ingredients tested here, showing a higher predictive capacity of these methods when compared to some others based on animal data (e.g. *in silico* models established using LLNA data). Corroborating this, previous studies have shown that these four assays have different predictive capacities in identifying skin sensitizers (1, 10, 39-42), demonstrating their potential to be applied in a testing approach as a way to overcome the technical limitations found in each technique.

According to the information declared on the labels of the ten henna products, two colorants only contained PPD in their composition. However, our chromatographic analyses identified PPD amounts in all products at different concentrations, ranging 0.03-4.3%, demonstrating undisclosed addition of this extreme contact allergen is performed in natural material-based cosmetics. Even though the levels of PPD do not exceed 6%, a limit set by the legislation of countries from South American trade bloc (MERCOSUR) for cosmetic products (43), the use of PPD is controversial from a human health perspective. In addition, there are geographical differences regarding PPD amounts acceptable in cosmetics. For instance, in Europe, PPD is permitted in concentrations up to 2% (44); whereas in the United States, where no testing or data regarding skin sensitization potential is required for cosmetics (3), no limits are provided for hair colorants (31). Thus, there is no world-wide consensus established for the tolerable limits of this chemical in cosmetics. It has been reported that individuals sensitized to PPD may react to this chemical at 0.0038% (21), which is a very low concentration in relation to the limits established by the legislations mentioned above and to those amounts found here for the henna products analyzed.

Regarding LAW, the levels found ranged at 0.041-0.518% in nine colorants. In one product, LAW was not detected, which suggests falsification since it should thus not be labeled as a henna-based product. Hazard assessment of skin sensitization of isolated LAW (ie, as pure substance) showed equivocal results in animal models (45), contrasting to the pure henna where contact allergy reactions are rarely reported. However, our *in vitro* findings identified LAW as being a potential contact allergen, demonstrating an associated hazard in

using this pure substance as a cosmetic ingredient. In fact, it is not recommended for use as a coloring agent in cosmetics in Europe due to its genotoxicity/mutagenicity potential *in vitro* and *in vivo* (45). LAW concentrations in henna can be up to 2%, from which it has been estimated that about 5% are bioavailable in the skin; thus low levels that provide a safe use of henna as a hair dye (25).

Given that, our data showed that all henna products tested were classified as skin sensitizers using different two-out-of-three ITSs, a conventional approach first introduced by Bauch et al (4). Interestingly, mDPRA or GARD skin classified the henna colorants as skin sensitizers in the same way as the combined outputs involving different endpoints, questioning the need for combined approaches. Among the techniques evaluated, previous studies showed that DPRA (accuracy = 80%) and GARD skin (accuracy = 86%) have reached the best accuracies by testing a wide range of chemical dataset (8, 40). Although no data have been published involving mixtures yet, it has been recently demonstrated that the combination of a high performing assay with less accurate techniques reduces the predictive capacity of a two-out-of-three ITS (46, 47). Corroborating of this, another study evaluated the predictive performances of DPRA and other two assays, namely KeratinoSens (accuracy 77%) and human cell line activation test (h-CLAT, accuracy 85%) based on skin sensitization KE-2 and 3, respectively (48). Through individual analysis as well as their binary combinations and the inclusion in a two-out-of-three ITS, the findings showed that the association of DPRA with h-CLAT has the best human correlation besides being cost-effective in comparison to the two-out-of-three approach (48). This highlights caution is necessary in selecting top performing methods, and in particular to perform defined approaches to testing and assessment products as established as part of an integrated approaches to testing and assessment (IATA) for skin sensitization (49).

In relation to the parameters investigated in this study, the hair dye ingredients and henna products tested yielded higher peptide depletion for cysteine than lysine. This peptide reactivity profile has been related to chemicals and mixtures with skin sensitization potential (32, 50). Furthermore, henna products containing PPD triggered a greater peptide reactivity and intracellular IL-18 production, when compared to the hair dye ingredients tested alone. Cases of severe allergic contact dermatitis after use of mixtures of henna and PPD in temporary holiday tattoos have been observed (26). Thus, it may be that the immune response to these “real-life” mixtures is even more complex due to combination effects. Therefore, PPD adulteration can change the toxicological profile of henna products. Corroborating these findings, literature has demonstrated that allergens within the mixtures, such as fragrances and hair dyes, can increase immunological events in an additive and/or synergistic manner (17).

In this context, the formation of reaction products, intermediates and possible by-products is expected during the hair dying process, since colorant formulations are complex mixtures of a wide range of substances. For instance, PPD can be involved in autoxidation and/or oxidation reactions with other ingredients (e.g. resorcinol and *m*-aminophenol, coupler agents which were observed on the label of some “henna cosmetics”), producing a series of reactive dimers and trimers or other reaction products (22, 51). Moreover, the manufacturers’ henna products for eyebrow coloring (products #8, #9 and #10) stated on the labels that, before application, the user needs mixed them with a solution containing hydrogen peroxide, an oxidizing agent whose reaction with PPD is well known to form possible allergen products, such as *p*-nitroaniline and 4,4-azodianiline (52). Although this condition (henna + hydrogen peroxide) was not investigated here, our study provides an analysis of the toxicological impact of the henna cosmetics adulterated with PPD as well as the presence of other synthetic ingredients declared on the label of some products. Thereby, the connection to contact allergy potential of the potentiation effects between henna and synthetic allergens added in the cosmetics and their reaction products formed cannot be discarded. In fact, this

may lead to a change of toxicological classification regarding skin sensitization hazard of these “natural product”-based cosmetics, as observed here.

Regarding the reference controls used, the surfactant SDS was classified as skin sensitizer by mDPRA and U-SENS, similar to data reported by the literature (1, 4). However, these results contrast to the human data, which report that SDS is a non-sensitizer. Therefore, as well as in LLNA, SDS is a false positive for mDPRA and U-SENS. This can be attributed to the fact that some surfactants, including SDS, can promote peptide dimerization, which would explain, in part, the discordant prediction in DPRA (4, 53). Also, U-SENS shows limitation for surfactants due to the possibility to cause cell membrane disrupting and then non-specific increase of CD86 expression, as highlighted by Piroird et al (1) through testing different surfactants (e.g. SDS, octanoic acid and Tween 80). In this case, the other assays tested (e.g. GARD skin, keratinocytes-associated IL-18 assay) seem to be suitable for surfactants as SDS.

Taken together, our findings highlight toxicological consequences of the undisclosed use of PPD in henna-based natural products, which may entail sensitization of susceptible consumers and ACD in previously sensitized individuals. Moreover, our hazard assessment showed that henna products containing PPD seem to be associated with a greater skin sensitization hazard when compared to the hair dye ingredients tested alone. Therefore, it is suggested that immune responses to these “real-life” mixtures may be complex in view of the associative effects between henna, added synthetic ingredients and possible derivatives formed during the hair dye process. Considering that the *in vitro* human-relevant tools to perform the skin sensitization hazard evaluation have been developed focusing pure substances only, further studies are also necessary to better understand their applicability for “real-life” mixtures.

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Tables

Table 1. Information declared on the label, and lawsone and *p*-phenylenediamine levels found in ten commercial henna-based hair coloring cosmetics, marketed in Brazil.

Products	Application	Origin	Ingredients declared on the label ^a				Levels (wt. %)	
			Henna	Other natural materials	PPD	Other synthetic materials	LAW	PPD
#1	Hair	Brazil	+				0.518 ± 0.003	1.091 ± 0.028
#2	Hair	Brazil	+	+	+	+	ND	2.970 ± 0.046
#3	Hair	Brazil	+	+		+	0.188 ± 0.001	0.030 ± 0.001
#4	Hair	Brazil	+	+		+	0.320 ± 0.002	0.032 ± 0.006
#5	Hair	Brazil	+	+			0.041 ± 0.001	4.321 ± 0.028
#6	Hair	Brazil	+	+			0.113 ± 0.001	1.020 ± 0.100
#7	Hair	France	+	+		+	0.359 ± 0.001	0.577 ± 0.015
#8	Eyebrow	Brazil	+	+	+	+	0.158 ± 0.001	2.541 ± 0.057
#9	Eyebrow	Brazil	+	+			0.103 ± 0.001	0.760 ± 0.017
#10	Eyebrow	India	+	+			0.032 ± 0.001	3.354 ± 0.163

Abbreviations: +, information declared on the label; ND, not detected; LAW, lawsone; PPD, *p*-phenylenediamine.

^aSee supplementary material (Table S1) for more details.

Table 2. Results of peptide reactivity assessment of hair dye ingredients and henna-based products.

Test materials	Solvent	mDPRA results				Prediction
		Cys-peptide depletion	Lys-peptide depletion	Mean depletion	Reactivity class	
Reference controls						
Glycerol (GLY)	Water	2.27 ± 3.14	0	1.13	Minimal	NS
Sodium dodecyl sulfate (SDS)	Water	0	95.50 ± 0.06	47.70	High	S
2,4-Dinitrochlorobenzene (DNCB)	Acetonitrile	100 ± 0	45.0 ± 9.47	72.50	High	S
Eugenol (EUG)	Acetonitrile	48.3 ± 2.73	24.53 ± 1.16	36.41	Moderate	S
Hair dye ingredients						
1,4-Diaminoanthraquinone (14D)	DMSO + acetonitrile	49.56 ± 3.35	2.80 ± 3.33	26.18	High	S
2-Amino-3-hydroxypyridine (2A3)	Water	100 ± 0	15.11 ± 1.65	57.56	High	S
Lawson (LAW)	DMSO + acetonitrile	100 ± 0	0.80 ± 0.80	50.40	High	S
5-Amino- <i>o</i> -cresol (5OC)	Acetonitrile	100 ± 0	19.41 ± 1.14	59.70	High	S
Hydroquinone (HQ)	Water	65.18 ± 11.06	81.90 ± 2.12	73.54	High	S
<i>p</i> -Phenylenediamine (PPD)	Acetonitrile	100 ± 0	51.72 ± 0.61	75.86	High	S
Resorcinol (RSC)	Water	13.70 ± 0.41	32.94 ± 0.20	23.32	Moderate	S
Disperse Orange 3 (DO3)	DMSO	84.13 ± 0.21	0	42.07	Moderate	S
Basic Red 51 (BR51)	Water	90.38 ± 5.19	0	45.19	High	S
Pyrogallol (PYRO)	Water	97.67 ± 0.24	29.62 ± 0.92	63.64	High	S
Henna-based hair coloring products						
#1	Methanol	95.94 ± 1.69	71.80 ± 11.19	83.87	High	S
#2	Methanol	99.13 ± 0.07	6.0 ± 2.06	52.56	High	S
#3	Methanol	96.53 ± 0.09	16.44 ± 1.61	56.48	High	S
#4	Methanol	95.24 ± 0.05	38.41 ± 6.42	66.83	High	S
#5	Methanol	92.30 ± 0.08	63.19 ± 10.14	77.75	High	S
#6	Methanol	98.63 ± 0.53	44.95 ± 1.41	71.79	High	S
#7	Methanol	99.15 ± 0.80	27.72 ± 8.28	63.44	High	S
#8	Methanol	99.64 ± 0.20	0	49.82	High	S
#9	Methanol	100 ± 0	0	50.0	High	S
#10	Methanol	98.05 ± 0.35	22.45 ± 10.95	60.25	High	S

Abbreviations: NS, non-sensitizer; S, sensitizer; mDPRA, micro-direct peptide reactivity assay.

Table 3. Results of CD86 expression assessment of hair dye ingredients and henna-based products by USENS™.

Test materials	Solvent	USENS™ results		
		CV ₇₀ (µg/mL)	EC ₁₅₀ (µg/mL)	Prediction
Reference controls				
Dimethyl sulfoxide (0.1%, DMSO)	-	-	-	NS
Glycerol (GLY)	Medium	> 200	ND	NS
Sodium dodecyl sulfate (SDS)	Medium	111.0	53.90	S
2,4-Dinitrochlorobenzene (DNCB)	DMSO	2.67	0.62	S
Eugenol (EUG)	DMSO	56.90	17.90	S
Hair dye ingredients				
1,4-Diaminoanthraquinone (14D)	DMSO	36.30	2.85	S
2-Amino-3-hydroxypyridine (2A3)	DMSO	165.0	2.67	S
Lawson (LAW)	DMSO	> 200	0.28	S
5-Amino- <i>o</i> -cresol (5OC)	DMSO	111.0	0.66	S
Hydroquinone (HQ)	Medium	19.20	0.93	S
<i>p</i> -Phenylenediamine (PPD)	DMSO	26.90	5.90	S
Resorcinol (RSC)	Medium	> 200	5.21	S
Disperse Orange 3 (DO3)	DMSO	> 200	2.05	S
Basic Red 51 (BR51)	Medium	15.70	3.69	S
Pyrogallol (PYRO)	Medium	33.89	10.09	S
Henna-based hair coloring products				
#1	Medium	> 200	40.56	S
#2	Medium	> 200	47.49	S
#3	Medium	> 200	ND	NS
#4	Medium	> 200	ND	NS
#5	Medium	70.10	4.41	S
#6	Medium	> 200	12.39	S
#7	Medium	> 200	72.56	S
#8	Medium	120.0	11.78	S
#9	Medium	> 200	87.15	S
#10	Medium	> 200	19.48	S

Abbreviations: NS – non-sensitizer; S – sensitizer; ND, not detected; CV₇₀, concentration which promote cell viability ≥ 70%; EC₁₅₀, concentration at which the test material induces a stimulation index of 150%.

Table 4. Results of CD86 expression assessment of hair dye ingredients by PBMDc test.

Test materials	Solvent	PBMDc test results		
		CV ₈₀ (µg/mL)	EC ₂₀ (µg/mL)	Prediction
Reference controls				
Dimethyl sulfoxide (0.1%, DMSO)	-	-	-	NS
Glycerol (GLY)	Medium	> 200	ND	NS
Sodium dodecyl sulfate (SDS)	Medium	60.75	ND	NS
2,4-Dinitrochlorobenzene (DNCB)	DMSO	2.13	0.92	S
Eugenol (EUG)	DMSO	> 200	ND	NS
Hair dye ingredients				
1,4-Diaminoanthraquinone (14D)	DMSO	37.95	ND	NS
2-Amino-3-hydroxypyridine (2A3)	DMSO	> 200	ND	NS
Lawson (LAW)	DMSO	> 200	ND	NS
5-Amino- <i>o</i> -cresol (5OC)	DMSO	> 200	ND	NS
Hydroquinone (HQ)	Medium	14.15	6.33	S
<i>p</i> -Phenylenediamine (PPD)	DMSO	16.20	ND	NS
Resorcinol (RSC)	Medium	> 200	ND	NS
Disperse Orange 3 (DO3)	DMSO	11.12	ND	NS
Basic Red 51 (BR51)	Medium	10.31	ND	NS
Pyrogallol (PYRO)	Medium	24.57	ND	NS

Abbreviations: NS, non-sensitizer; S, sensitizer; ND, not detected; CV₈₀, concentration which promote cell viability ≥ 80%; EC₂₀, concentration at which the test material induces a ΔCD86 = 20%.

Table 5. Summary of the predictions of the seven *in vitro* assays evaluated for skin sensitization hazard assessment of hair dye ingredients and their concordance in relation to animal and human data.

Test materials	CAS no.	Human classification ¹	Animal classification ²	<i>In vitro</i> prediction						
				mDPRA	IL-18	USENS	IL-8 intra	IL-8 extra	PBMDC	GARD skin
Reference controls										
Dimethyl sulfoxide (0.1%, DMSO)	67-68-5	Negative ^a	Negative ^a	NS	NS	NS	NS	NS	NS	NS
Glycerol (GLY)	56-81-5	Negative ^b	Negative ^h	NS	NS	NS	NS	NS	NS	NS ^s
Sodium dodecyl sulfate (SDS)	151-21-3	Negative ^c	Weak ^c	S	NS	S	NS	NS	NS	NS ^{s,t,u}
2,4-Dinitrochlorobenzene (DNCB)	97-00-7	Positive ^c	Extreme ^c	S	S	S	S	S	S	S ^{s,t}
Eugenol (EUG)	97-53-0	Positive ^c	Weak ^c	S	S	S	S	S	NS	S ^s
2-Hydroxyethyl acrylate (2HA)	818-61-1	Positive ^d	Moderate ^d	-	-	-	-	-	-	S
Hair dye ingredients										
1,4-Diaminoanthraquinone (14D)	128-95-0	Positive ^e	Moderate ⁱ	S	S	S	NS	NS	NS	S
2-Amino-3-hydroxypyridine (2A3)	16867-03-1	Positive ^e	Negative ^j	S	S	S	S	S	NS	S
Lawson (LAW)	83-72-7	NA	Equivocal ^k	S	NS	S	S	S	NS	S
5-Amino- <i>o</i> -cresol (5OC)	2835-95-2	Positive ^e	Moderate ^l	S	NS	S	S	S	NS	S
Hydroquinone (HQ)	123-31-9	Positive ^f	Strong ^m	S	S	S	S	S	S	S ^{t,u}
<i>p</i> -Phenylenediamine (PPD)	106-50-3	Positive ^g	Extreme ⁿ	S	S	S	S	NS	NS	S
Resorcinol (RSC)	108-46-3	Positive ^e	Strong ^o	S	S	S	NS	S	NS	S ^r
Disperse Orange 3 (DO3)	730-40-5	Positive ^g	Very weak ^p	S	S	S	NS	NS	NS	S
Basic Red 51 (BR51)	77061-58-6	NA	Negative ^q	S	S	S	NS	NS	NS	S
Pyrogallol (PYRO)	87-66-1	Positive ^f	Negative ^r	S	S	S	NS	S	NS	S
Concordance vs. Human data			78.5%	91.7%	92.3%	92.3%	69.2%	76.9%	38.5%	100%
Concordance vs. Animal data				76.9%	64.3%	78.6%	64.3%	57.1%	50%	73.3%

Abbreviations: NS, non-sensitizer; S, sensitizer; NA – data not available by literature; CAS No – Chemical Abstracts Service Number.

¹Classification based on the human repeated insult patch test (HRIPT).

²Classification based on murine local lymph node assay (LLNA) and/or guinea pig maximization test (GPMT)/Buehler test (BT).

^aReference 54; ^bReference 55; ^cReference 56; ^dReference 57; ^eReference 58; ^fReference 12; ^gReference 59; ^hReference 60; ⁱReference 61; ^jReference 62; ^kReference 45; ^lReference 63; ^mReference 64; ⁿReference 65; ^oReference 66; ^pReference 67; ^qReference 68; ^rReference 69; ^sReference 36; ^tReference 38; ^uReference 37.

Table 6. Summary of *in vitro* predictivities of single assays and their different combinations based on a two-out-of-three strategy.

Henna-based hair coloring products	<i>In vitro</i> prediction							
	Single assays				Combination of assays (two-out-of-three strategies)			
	mDPRA	IL-18	USENS	GARD skin	mDPRA + IL-18 + USENS	mDPRA + IL-18 + GARD skin	mDPRA + USENS + GARD skin	IL-18 + USENS + GARD skin
#1	S	S	S	S	S	S	S	S
#2	S	S	S	S	S	S	S	S
#3	S	S	NS	S	S	S	S	S
#4	S	S	NS	S	S	S	S	S
#5	S	S	S	S	S	S	S	S
#6	S	S	S	S	S	S	S	S
#7	S	NS	S	S	S	S	S	S
#8	S	NS	S	S	S	S	S	S
#9	S	S	S	S	S	S	S	S
#10	S	S	S	S	S	S	S	S

Abbreviations: NS, non-sensitizer; S, sensitizer.

Figure legends

Figure 1. Effects of hair dye ingredients **(A)** and henna-based products **(B)** on intracellular IL-18 production by HaCaT keratinocytes. Cells (1.5×10^5 cells/mL) were seeded into a 24-well flat plate and incubated overnight. After that, the cells were exposed to culture medium only (control cells), non-cytotoxic concentration of each test materials. After 24 h of exposure, cell lysates were obtained to quantify IL-18 levels. The following numbers in parentheses represent the non-cytotoxic concentrations ($\mu\text{g/mL}$) tested for each reference control and hair dye ingredient: SDS (64), GLY (365), DNCB (2), EUG (26), 14D (1), 2A3 (324), LAW (110), 5OC (15), HQ (17), PPD (40), RSC (288), DO3 (10), BR51 (3) and PYRO (60). Regarding henna-based hair coloring products, the concentrations tested were as follows: #1 (91.7), #2 (82.3), #3 (621), #4 (305), #5 (46), #6 (50.1), #7 (56.7), #8 (1.31), #9 (134) and #10 (233). Data represent the mean $\pm$ SD of three independent experiments. (* $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$ vs. control or DMSO (0.1%). ANOVA followed by Dunnett's test).

Figure 2. Effects of hair dye ingredients on intracellular and extracellular IL-8 levels in U937 cells. Cells (2.5×10^5 cells/mL) were exposed to non-cytotoxic concentration of each hair dye ingredient for 45 h. After that, the cell lysates and supernatants were obtained for measurement of intracellular **(A)** and extracellular **(B)** IL-8 levels, respectively. The following numbers in parentheses represent the non-cytotoxic concentrations ($\mu\text{g/mL}$) tested for each reference control and hair dye ingredients: SDS (53.9), GLY (200), DNCB (0.6), EUG (17.9), 14D (2.9), 2A3 (2.7), LAW (0.3), 5OC (0.7), HQ (0.9), PPD (5.9), RSC (5.2), DO3 (2.1), BR51 (3.7) and PYRO (10.1). Data represent the mean $\pm$ SD of three independent experiments. (* $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$ vs. control or DMSO (0.1%). ANOVA followed by Dunnett's test).

Figure 3. Effects of hair dye ingredients and henna-based products of transcriptional levels of 200 genomic biomarkers, referred as the GARD skin prediction signature. MUTZ-3-derived cells (2×10^5 cells/mL) were seeded in 12-well plates and exposed to each test material for 24 h. After that, cells were lysed for total RNA extraction and gene expression analysis performed using a digital bar-coding platform (NanoString Technologies). The following numbers in parentheses represent the non-cytotoxic concentrations ($\mu\text{g/mL}$) tested for each reference control and hair dye ingredient: 2HA (12), 14D (5), 2A3 (55), LAW (87), 5OC (62), PPD (8), DO3 (10), BR51 (3) and PYRO (6). Regarding henna-based hair coloring products, the concentrations tested were as follows: #1 (20), #2 (80), #3 (100), #4 (100), #5 (40), #6 (40), #7 (80), #8 (55), #9 (100) and #10 (100). A test material was classified as a skin sensitizer when the support vector machine (SVM) median output value of the three independent replicates > 0 .

Figures

Figure 1.

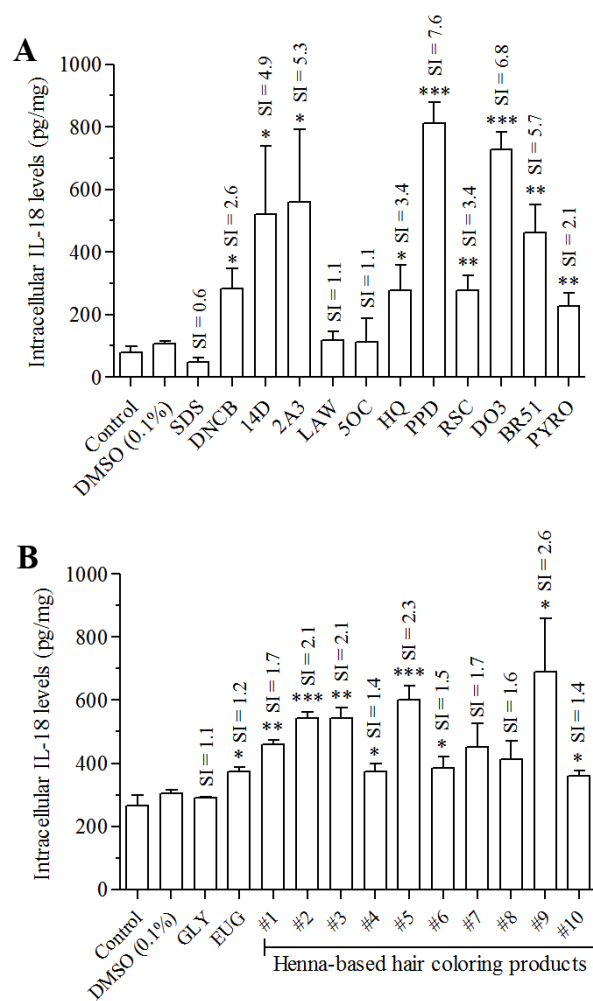


Figure 2.

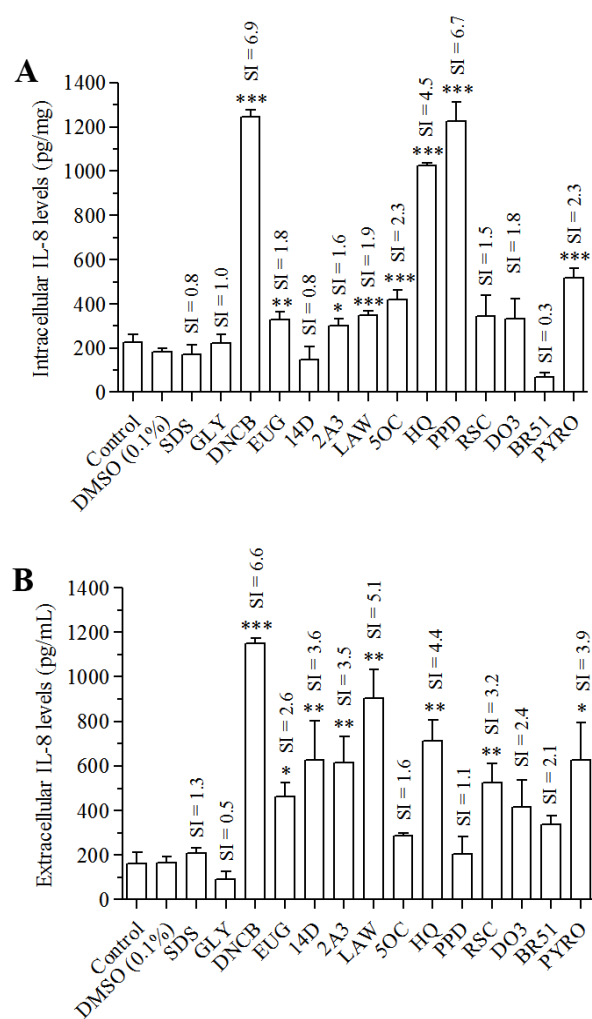


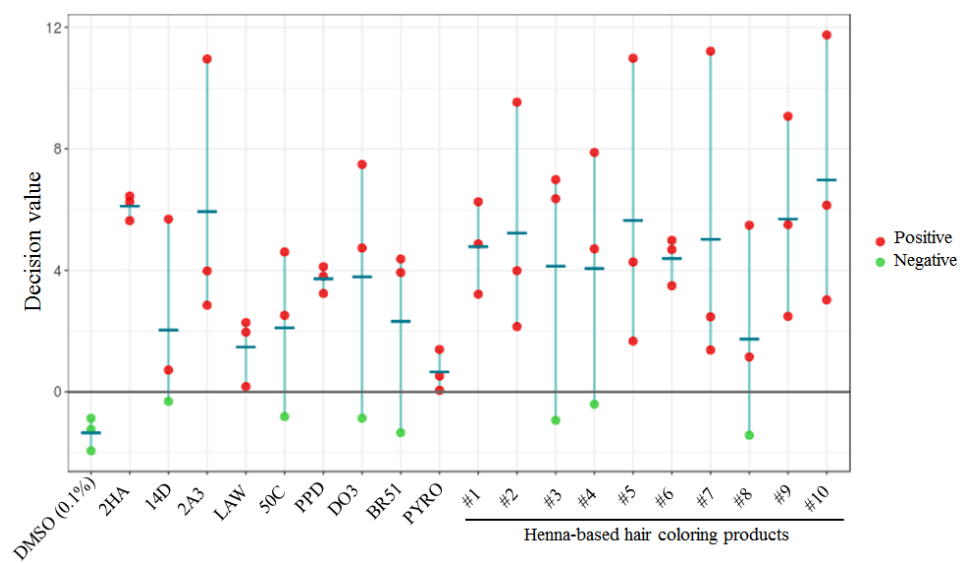
Figure 3.

Table S1. Ingredients declared on label of the henna (*Lawsonia inermis*)-based hair coloring cosmetics marketed in Brazil. The ingredients are presented in the order of greatest amount present in the product, although the amount of each one is not supplied by the manufacturers.

Products	Color	Ingredients declared on the label ^a
#1	Black for dark hair or reddish brown for light hair	Henna extract
#2	Black	Henna and <i>Zea mays</i> extracts, sodium perborate , <i>m</i>-phenylenediamine , <i>p</i>-phenylenediamine (PPD) and resorcinol
#3	Brown	Henna, <i>Zea mays</i> and <i>Ilex paraguariensis</i> extracts, HC Red no. 3 , HC Blue no. 2 and HC Yellow no. 2
#4	Copper	Henna, <i>Zea mays</i> and <i>Ilex paraguariensis</i> extracts, HC Red no. 3 , HC Blue no. 2 and HC Yellow no. 2
#5	Champagne blonde	Henna, <i>Phyllanthus emblica</i> , <i>Acacia concinna</i> , <i>Indigofera tinctoria</i> and <i>Terminalia bellerica</i> extracts
#6	Red wine	Henna, <i>Phyllanthus emblica</i> , <i>Acacia concinna</i> , <i>Indigofera tinctoria</i> and <i>Terminalia bellerica</i> extracts
#7	Red	Henna, <i>Lawsonia alba</i> , <i>Juglans regia</i> , <i>Matricaria chamomile</i> , <i>Indigofera tinctoria</i> , <i>Curcuma longa</i> and <i>Bixa orellana</i> extracts and picramate
#8 ^b	Dark blonde	Henna extract, sodium perborate , carboxymethyl cellulose , <i>p</i>-phenylenediamine (PPD) sulfate , sodium sulfate , tartaric acid , <i>m</i>-aminophenol , <i>p</i>-aminophenol , disodium lauryl sulfosuccinate , magnesium stearate , algin and <i>Sapindus mukurossi</i> peel extract
#9 ^b	Dark blonde	Henna and <i>Indigofera tinctoria</i> extracts, <i>Phyllanthus emblica</i> fruit powder, <i>Eclipta prostrata</i> leaf powder, <i>Azadirachta indica</i> bark/seed extract, <i>Centella asiatica</i> leaf extract, <i>Terminalia chebula</i> fruit powder, <i>Terminalia bellerica</i> fruit powder, Moroccan lava clay, <i>Curcuma longa</i> root powder, <i>Cassia italica</i> leaf powder and <i>Vetiveria zizanioides</i> root powder
#10 ^b	Reddish brown	Henna leaf extract, <i>Hibiscus rosa-sinensis</i> flower powder, <i>Azadirachta indica</i> bark/seed extract, <i>Phyllanthus emblica</i> fruit powder and <i>Eclipta prostrata</i> leaf powder

^aSynthetic materials declared on the label are highlighted in bold.

^bThese products are used mixed with hydrogen peroxide.

Table S2. GARDTM skin prediction based on the median of the support vector machine (SVM) decision values.

Test materials	SVM median output value	Prediction (S if median > 0)
Reference controls		
Dimethyl sulfoxide (0.1%, DMSO)	-1.34	NS
2-Hydroxyethyl acrylate	6.11	S
Hair dye ingredients		
1,4-Diaminoanthraquinone (14D)	2.03	S
2-Amino-3-hydroxypyridine (2A3)	5.93	S
Lawsone (LAW)	1.47	S
5-Amino- <i>o</i> -cresol (5OC)	2.10	S
<i>p</i> -Phenylenediamine (PPD)	3.72	S
Disperse Orange 3 (DO3)	3.78	S
Basic Red 51 (BR51)	2.32	S
Pyrogallol (PYRO)	0.65	S
Henna-based hair coloring products		
#1	4.78	S
#2	5.22	S
#3	4.13	S
#4	4.06	S
#5	5.64	S
#6	4.39	S
#7	5.02	S
#8	1.73	S
#9	5.68	S
#10	6.97	S

Abbreviations: NS, non-sensitizer; S, sensitizer.

Fig. S1. Representative chromatograms of the HPLC analyses of the henna-based hair coloring products for detection of *p*-phenylenediamine (PPD) and lawsone.

