Are coffee silverskin extracts safe for topical use? An in vitro and in vivo approach

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ABSTRACT

Recent changes in regulatory requirements and social views on animal testing have incremented the development of reliable alternative tests for predicting skin and ocular irritation potential of products based on new raw materials. In this regard, botanical ingredients used in cosmetic products are among those materials, and should be carefully reviewed concerning the potential presence of irritant con-stituents. In particular, cosmetic products used on the face, in vicinity of the eyes or that may come in contact with mucous membranes, should avoid botanical ingredients that contain, or are suspected to contain, such ingredients. In this study, we aimed to evaluate the effect of a new cosmetic ingredient, namely, coffee silverskin (CS), with an in vitro skin and ocular irritation assay using reconstructed human epidermis, EpiSkin™, and human corneal epithelial model, SkinEthics™ HCE, and an in vivo assay. Three different extracts of CS were evaluated. The histology of the models after extracts applica-tions was analysed. The in vitro results demonstrated that extracts were not classified as irritant and the histological analyses proved that extracts did not affect both models structure. The content of caffeine, 5-hydroxymethyl furfural and chlorogenic acid was quantified after the epidermal assay. The in vivo test carried out with the most promising extract (hydroalcoholic) showed that, with respect to irritant effects, these extracts can be regarded as safe for topical application.

Keywords:
In vitro skin cultures, Interleukin-1α (IL-1α), Skin irritation potential, Coffee silverskin, EpiSkin™
SkinEthics™ HCE

1. Introduction

In recent years, sustainable approaches have become an essen-tial challenge for different industries. Answering to this issue, the present study was undertaken to investigate the potential use of coffee silverskin (CS) extracts in cosmetic products. Usually, per coffee fruit are found two coffee beans, each one covered by a thin closely skin called silverskin (Saenger et al., 2001). CS is a main by-product of the coffee roasting procedure and has no commercial value, being discarded as a solid waste (Saenger et al., 2001). This may have negative effects on the environment requiring proper management. Recent advances in industrial biotechnology leads to potential opportunities for economic valorization of this by-product (Pandey et al., 2000). Some work has been performed on the properties of CS, in particular its antioxidant behaviour, which reveals a good content (Borreli et al., 2004; Costa et al., 2014; Narita and Inouye, 2012; Panusa et al., 2013; Pourfarzad et al., 2013; Rodrigues et al., 2015). An example is chlorogenic acid (CGA), which is a highly valuable natural polyphenol com-pound used in medicine and industries (Sato et al., 2011). However, some process steps in coffee production could affect CGA content, such as roasting, decaffeination and/or blending (Mills et al., 2013). Also, the caffeine content should be very high and similar to that of coffee beans (Bresciani et al., 2014; Narita and Inouye, 2012;
Pourfarzad et al., 2013). These compounds are believed to provide in vivo protection against free radical damage. As coffee beans, CS contains several classes of health compounds such as phenolics, diterpenes, xanthines, and vitamin precursors (Alves et al., 2009; Ludwig et al., 2012). Caffeine is being increasingly used in cosmetics due to its high biological activity and ability to penetrate the skin barrier (Herman and Herman, 2013). A number of claims, as anti-inflammatory properties, are based on the implicit assumption that this bioactive substance is effectively released from the formulation and probably through epidermis into the dermal and subcutaneous tissues, preventing the excessive accumulation of fat in cells, and providing a slimming effect (Bolzinger et al., 2008; Herman and Herman, 2013). This alkaloid stimulates the degradation of fats during lipolysis through inhibition of the phosphodiesterase activity and has potent antioxidant properties (Herman and Herman, 2013). However, studies assessing the skin absorption of caffeine released from extracts are extremely rare in the literature. Another compound that should be taken into consideration regarding CS is 5-hydroxymethyl furfural (HMF), which is formed during coffee roasting (del Campo et al., 2010). HMF is cytotoxic, irritating to the eyes, skin and mucous membranes at high concentrations, being very important to quantify its presence in CS extracts (Capuano and Fogliano, 2011).

The assessment of irritation is one of the primary procedures to evaluate and hazard classify a substance, particularly, in cosmetics or pharmaceuticals (Cotovio et al., 2010). In line with the 7th amendment deadlines, European Union bans the in vivo skin irritation assessment on ingredients for cosmetic purposes, regarding concerns about the test’s reproducibility, plus animal welfare and political pressures (Draize et al., 1944; Spielmann et al., 2007). A number of in vitro tests to assess potential skin or eye irritants have been developed. The reconstructed human epidermis EpiSkin™ test method was validated by European Union Reference Laboratory for alternatives to animal testing (ECVAM) as replacement test for the prediction of acute skin irritation. According to Cotovio et al. (2010), the assessment of ocular irritation is also one of the primary procedures to evaluate and hazard classify a new substance. The in vivo Draize irritation rabbit eye test continues to be described in the current OECD test guidelines, but ethical questions are leading to the development of in vitro alternative tests, such as Human Corneal Epithelial Model (SkinEthic™ HCE), which are still under validation by ECVAM.

The aim of this study is to evaluate the in vitro and in vivo irritant potential of three CS extracts. For both EpiSkin™ and SkinEthic HCE™ assays, MTT and IL-1α were used as endpoints. After the extract contact with the model, the histology of the model was evaluated. To ensure the possible content of caffeine, COA and HMF that pass through the RHE, an HPLC assay was developed. In vivo skin irritation potential observed after single application under occlusion was assessed along with sodium lauryl sulfate (SLS) solution (2%, w/v) as irritant model (positive control) and water as non-irritant (negative control).

2. Materials and methods

2.1. Chemicals

Coffee silverskin samples were provided by a national coffee roaster (BICAFÉ – Torrefação e Comércio de Café Ltda, Portugal). EpiSkin™ and SkinEthic™ HCE model were purchased from SkinEthic Laboratories (Lyon, France). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and sodium lauryl sulfate (SLS; purity > 99%) were purchased from Sigma–Aldrich Chemical (St Quentin Fallavier, France). ELISA Quantikin kit for IL-1α measurements was from R&D Systems (Lille, France).

Deionised water was obtained using Milli-Q water purification system (TGI Pure Water Systems, USA). Ethanol was obtained from Panreac (Barcelona, Spain). HPLC-grade acetonitrile was from Fluka (Madrid, Spain). 5-(Hydroxymethyl)furfural (HMF), caffeine and chlorogenic acid (CGA) standards were from Sigma–Aldrich (Stein-heim, Germany). Metaphosphoric acid, isopropanol, phosphate buffered saline (PBS; pH 7.4) and paraformaldehyde were from Sigma–Aldrich (St. Louis, Missouri, USA).

2.2. Preparation of extracts

CS was milled to particle size of approximately 0.1 mm using a 11 basic analysis mill (IKA Wearhe, Staufen, Germany) and stored in silicone tubes at room temperature (25–28°C) until extract preparation, as described by different authors (Costa et al., 2014; Rodrigues et al., 2015). Samples (1 g) were submitted to solvent extraction by maceration with 20 mL of water, ethanol:water (1:1, v/v) or ethanol for 30 min at 40°C. The three different extracts obtained were filtered through Whatman No. 1 paper filter and the filtrates collected (Costa et al., 2014).

2.3. In vitro models assays

In order to determine the irritant potential of CS extracts on skin and eye, in vitro tests were performed. Three different extracts mentioned above were tested.

The EpiSkin™ model [large model] was obtained from a standardized large-scale production certified by the ISO9001 standard. EpiSkin™ units were delivered to the laboratory within 24 h. Upon arrival, tissues were transferred to 12 well plates containing 37°C pre-warmed maintenance media (2 mL/well) and incubated overnight at 37°C, 5% CO₂ and 95% relative humidity.

The SkinEthic™ HCE model is composed of immortalized human corneal epithelial cells cultured in a chemically defined medium and seeded on a synthetic membrane at the air-liquid interface. The tissue is represented by a multilayered epithelium, with five to seven cell layers and a surface area of 0.5 cm². The assay was performed according to the manufacturer instructions (Alépée et al., 2013; Cotovio et al., 2010). Upon arrival, tissues were transferred to 24 well plates containing 37°C pre-warmed maintenance media (1 mL/well) and incubated 24 h at 37°C, 5% CO₂ and 95% relative humidity.

2.3.1. Skin irritation test

The in vitro reconstructed human skin tissue (EpiSkin™ method, proposed to replace animal testing for skin corrosivity and skin irritation (Tornier et al., 2010), is based on determining cell viability, and cytokine release (IL-1α) as an additional endpoint. The reconstructed 0.38 cm² skin inserts were used and the assay was performed according to the manufacturer instructions and protocol. Negative (PBS-treated) and positive controls (5% w/v of SLS) were used. After exposing to the extracts and controls for 15 min, the epidermis samples were washed with sterile PBS and then incubated in the maintenance medium. After 42 h, the medium was collected and frozen at ~20°C for further determination of IL-1α. Cell viability was determined by the MTT assay. Viability was calculated, considering 100% for the negative control. The histology of models was also evaluated as described below.

2.3.2. Ocular irritation test

The SkinEthic™ HCE model was used and the assay performed according to the manufacturer instructions and protocol. Negative (PBS-treated) and positive controls (ethanol) were used. After exposing to the extracts and controls for 10 min, the epidermis samples were washed with sterile PBS and then incubated in the
maintenance medium for 16 h. After this time, the medium was collected and frozen at −20 °C for further determination of IL-1α, and the cell viability was determined by the MTT assay. Viability was calculated, considering 100% for the negative control. The model was also analysed regarding the histological aspects.

2.3.3. MTT assay

Immediately after rinsing, the tissues were evaluated for cell viability using the MTT assay, where yellow MTT is reduced to purple formazan primarily by enzymes (reductases) located in the mito-chondria of living cells. A stock solution of MTT was prepared in maintenance media (provided with tissues) just prior to use and warmed to 37 °C in a water bath. Tissues were transferred to 24-well plates (Corning Inc., Corning, New York) containing 300 μL MTT medium per well and placed in the 37 °C, 5% CO₂ humidified incubator. After 3 h incubation, the tissues were removed from the MTT medium and any residual MTT media on the exterior of the tissue was blotted with absorbent pads. The formazan salt was extracted from the tissues by transferring them to 24-well plates containing isopropanol. The submerged tissues were incubated 3 h at room temperature protected from light. The plates were shaken for approximately 15 min once the extraction was complete. The optical density of the extracted formazan was determined by transferring 200 μL of each extraction solution into a clean 96-well plate. Isopropanol was used as a blank. The plates were read using a spectrophotometer (Synergy HT Microplate Reader; BioTek Instru- ments, Inc., Winooski, VT, USA) at 570 nm. Relative cell viability was calculated for each tissue as percent of the mean of the negative control tissues.

For both models, if the percentage of viability was >50%, the substance was predicted as Non-Irritant (EU classification: no label; GHS classification: No category); if the percentage of viability was ≤50%, the substance was predicted as Irritant (EU classification: R38, R41 or R36, depending on the model; GHS classification: Cat-egory 1 or Category 2) (Cotovio et al., 2010).

2.3.4. IL-1α assay

For assessing the release of IL-1α, the culture media underneath the tissues were collected at the end of the post-incubation period and kept at −20 °C until measurement. The quantification of IL-1α was performed using the commercial enzyme-linked immunosor-bent assay (ELISA) kit Quantikine®DLSAO (R&D Systems, UK) according to the manufacturer’s instructions. Mean concentrations (pg/mL) were obtained using duplicate measurements per tissue. IL-1α released was expressed as absolute data.

2.3.5. Histology

After incubating in the presence of CS extracts, EpiSkin™ and SkinEthic™ HCE samples (three samples per extract) were fixed with 2% paraformaldehyde during 1 h and embedded in Richard-Allan Scientific™ Neg-50™ Frozen Section Medium (ThermoScientific™) before cryosections (7 μm) preparation (Microm HM550 cryostat; ThermoScientific™). Samples were stained with haematoxylin–eosin–safran (H&E), and epidermal thickness was measured using image analysis with Axiosvision software (Zeiss, Sartrouville, France). Each image was studied for changes to the epidermis and especially to cell morphology.

2.4. Analysis of caffeine, HMF and CGA content in EpiSkin™

The HPLC method used in this study to quantify HMF, CGA and caffeine in CS extracts was based on the work of Lemos et al. (2010) and Rivelli et al. (2007). The chromatographic analysis was performed in a HPLC integrated system (Jasco, Japan) equipped with a PU-980 pump and a Jasco AS-950 automatic sampler with a 20 μL loop. Detection was performed with a Jasco model MD-2010 multiwavelength diode-array detector (DAD). The column employed was a Luna SU C18 (5 μm, 150 mm × 4.60 mm) chromatographic column (Teknokroma, Spain). The mobile phase adopted was aqueous metaphosphoric acid solution:acetonitrile (82:18, v/v) with a flow rate of 1 mL/min and a column temperature of 23 °C. Analytes were monitored at 275 nm, 285 nm and 330 nm, respectively, for caffeine, HMF and CGA, and quantification was performed on the basis of the internal standard method. Chromatographic data were analysed using the ChromNAV Software (Jasco, Japan). Calibration curves were prepared for HMF (0.36–93.0 mg/mL), chlorogenic acid (5.0–160.0 mg/mL) and caffeine (4.25–136.0 mg/mL). Analyses were carried out in duplicate. Confirmation of compounds identities was performed by comparing retention times and co-elution with authentic standards and also by UV absorption spectra.

2.5. In vivo skin irritation

2.5.1. Patch test

A single blinded study was done in order to evaluate the in vivo skin irritation. Twenty healthy individuals (15 women and 5 men) with a mean age of 30 ± 5 years, without known dermatological dis-eases or allergy to substances in topical products, participated in a blind study, in accordance with Declaration of Helsinki. Informed consent was obtained from all volunteers. The volunteers were asked to not apply any topical products in the forearms 24 h before the beginning and throughout the test period. Additionally, solar exposure was forbidden. Along with extracts, SLS (2%, w/v, aqueous solution) and purified water were also assayed, respectively, as positive and negative control. Five sites were marked in the inner forearms of volunteers. Before application of patch tests, the areas on the ventral part of each forearm were marked using a skin marker, and basal values were obtained by non-invasive measuring method. After basal measures were taken, the patches were applied. Fifty microlitres of the test solutions were applied on a filter paper disc (12 mm, Filter Paper Discs, SmartPractice, Phoenix, USA) and occlusion was achieved with aluminium cham-bers (12 mm, Finn Chambers, SmartPractice, Phoenix, USA). Patches were removed after 48 h, and following a period of 2 h of observa-tion patch test areas were evaluated by non-invasive measuring methods and visualscoring.

2.5.2. Visual and instrumental assessments

The visual assessment of the degree of irritation was graded according to the following scale, previously used by Agner et al. (2000): 0 – no reaction; 1 – weak, spotty erythema; 2 – well perceptible erythema covering the total exposure area; 3 – moderate erythema or severe erythema that covers the total exposure area. The same scale was used for oedema: 0 – no reaction; 1 – weak, spotty oedema; 2 – well perceptible oedema covering the total exposure area; 3 – moderate or severe oedema that covers the total exposure area (Agner et al., 2000).

Non-invasive biophysical measurements were also performed. All measurements were made with controlled temperature (21–22 °C) and relative humidity (45–55%). Transepidermal water loss (TEWL) is traditionally used to assess skin barrier function (Wa and Maibach, 2010). Barrier function was evaluated by TEWL measurements, carried out with a Tewameter® TM 210 (Courage Khazaka, Electronic GmbH, Cologne, Germany), which measures the relative humidity percentage which diffuses from the dermis to the skin surface (g/cm² h).

2.6. Statistical analysis

Data were reported as mean ± standard deviation of at least triplicate experiments. Statistical analysis of the results was performed.
with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different extracts for all assays. Post hoc comparisons were performed according to Tukey’s HSD test. In all cases, $p < 0.05$ was accepted as denoting significance.

3. Results and discussion

3.1. Skin irritability tests

Irritation is defined as the "production of reversible damage to the skin following the application of a test substance for up to 4 h," therefore, the more significant the initial injury (cell death), the stronger the irritant effect (UNICEF, 2011). In order to confirm the potential of the extracts for topical use, irritability assays with EpiSkin™ test were performed. EpiSkin™ is an alternative in vitro method efficient in predicting epidermal irritation in vivo caused by irritants. Data for the cell viability and the IL-1α release for aqueous, hydro-alcoholic (WA) and alcoholic (A) extracts of CS medium after EpiSkin™ assay.

### Table 1

<table>
<thead>
<tr>
<th>Extract</th>
<th>Viability (%)</th>
<th>IL-1α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>117.7 ± 11.7a</td>
<td>8.5 ± 0.1a</td>
</tr>
<tr>
<td>WA</td>
<td>105.9 ± 15.9a</td>
<td>76.4 ± 2.0b</td>
</tr>
<tr>
<td>A</td>
<td>117.8 ± 9.8a</td>
<td>17.6 ± 3.2a</td>
</tr>
<tr>
<td>PC (SLS)</td>
<td>11.6 ± 4.2b</td>
<td>522.9 ± 32.1c</td>
</tr>
<tr>
<td>NC(PBS)</td>
<td>100 ± 9.7a</td>
<td>28.4 ± 2.6a</td>
</tr>
</tbody>
</table>

Water (W), hydro-alcoholic (WA) mixture, alcohol (A), a positive control (PC: SLS = sodium lauryl sulfate) and a negative control (NC: PBS = phosphate buffered saline) were used. Values are expressed as mean ± SD (n = 3). Values in the same column followed by different letters indicate significant differences ($p < 0.05$).

### Table 2

<table>
<thead>
<tr>
<th>Extract</th>
<th>HMF (µg/mL extract)</th>
<th>CGA (µg/mL extract)</th>
<th>Caffeine (µg/mL extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>2.28 ± 0.18</td>
<td>–</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td>WA</td>
<td>2.39 ± 0.27</td>
<td>–</td>
<td>1.54 ± 0.14</td>
</tr>
<tr>
<td>A</td>
<td>2.26 ± 0.09</td>
<td>–</td>
<td>1.40 ± 0.07</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD (n = 3).

Significant difference ($p < 0.05$).

### Table 2

Quantification of HMF, CGA and CAF in aqueous (W), hydro-alcoholic (WA) and alcoholic (A) extracts of CS medium after EpiSkin™ assay.

The assessment of ocular irritation is part of the early testing procedure for the evaluation of new cosmetic ingredients (Cotovio et al., 2010). The use of cosmetic products can produce adverse effects on the ocular surface, ranging from mild discomfort to vision-threatening conditions (Coroneo et al., 2006). These complications can be related to allergy or toxicity (Coroneo et al., 2006).
In this field, numerous non-animal test systems have been developed over the years (OECD, 2009a,b). SkinEthic™ HCE is one of the two reconstructed human tissue that are currently available for the purpose of eye irritation and disqualification by ECVAM (Alépée et al., 2013).

In order to evaluate the eye irritation potential of CS extracts, a SkinEthic™ HCE test was done. Table 3 summarizes the results for cell viability and release of IL-1α observed with extracts and controls.

The viability of cells with aqueous and hydro-alcoholic extracts were higher than 50%, with percentages of 132.6 ± 21.3 and 123.6 ± 13.7, respectively, which means that the extracts were classified as non-irritants for the eyes. Ethanol extract presented a percentage of viability of 48.0 ± 7.5, being in the borderline between irritant and non-irritant, which is justify by the higher amount of alcohol used. Regarding the IL-1α release, no significant differences were observed between negative control and aqueous or hydro-alcoholic extracts. However, the release of IL-1α slightly
increased when using the positive control and ethanol extract as compared to the negative control. As mentioned by manufacture, ethanol was used as negative control.

The histological images obtained after topical application of the aqueous and hydro-alcoholic extracts of CS also confirms that both are not irritant, being good candidates for cosmetic ingredients. Regarding alcoholic extract, results are very different. As it is possible to confirm, this extract leads to histological alterations in SkinEthic™ HCE tissues, similarly to positive control (Fig. 3).

As far as we know, this is also the first time that SkinEthics™ HCE is used to evaluate the ocular irritation for food by-products extracts in order to incorporate them in cosmetics as active ingredients.

3.4. In vivo test

According to Miles et al. (2014), reconstructed human epidermis like EpiSkin™ has some limitations. This model is composed of a highly differentiated multi-layer human epidermis cells, but are totally lacking in any network of the pilo-sebaceous units, which regulate dermal homeostasis (Miles et al., 2014). Also, the hydrolipid film cannot be totally reproduced regarding its bio-physiological properties of components (urea, glycolic acid) neither to the occlusive effect of the film’s lipid component (ceramides) (Miles et al., 2014). Some cells normally present in human skin are not present in this model such as dendritic Langerhans’ cells. The higher penetration rate of these tissues makes these skin models more sensitive to test compounds (Perkins et al., 1999), which could result in over-prediction of strong irritants (Wellis et al., 2004). Thus, to ensure that the extract is completely safe, some other assays like patch test should be done. Patch testing after a single application is a widely used procedure to evaluate acute irritant reactions in human volunteers.

The previous assays led us to select the hydro-alcoholic extract as the best one to be incorporated in cosmetic products, as it did not affect the morphology of skin and ocular models, with high cell viability for both assays and low IL-1α release. Also the CAF content was higher than in the other extracts evaluated and, according to Rodrigues et al. (2015), the antioxidant content and antimicrobial activity is higher. Considering not only these results but also sustainability questions and other previous works regarding the antioxidant activity of these extracts, hydro-alcoholic extract seems to be the best one, being selected for the final patch test (Bresciani et al., 2014; Costa et al., 2014; Narita and Inouye, 2012).

Table 3

<table>
<thead>
<tr>
<th>Extract</th>
<th>Viability (%)</th>
<th>IL-1α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>132.6 ± 21.3a</td>
<td>41.4 ± 2.5a</td>
</tr>
<tr>
<td>WA</td>
<td>123.6 ± 13.7a</td>
<td>34.2 ± 1.6a</td>
</tr>
<tr>
<td>A</td>
<td>48.0 ± 7.5b</td>
<td>82.2 ± 22.9b</td>
</tr>
<tr>
<td>PC (ethanol)</td>
<td>2.9 ± 0.5c</td>
<td>114.4 ± 2.5c</td>
</tr>
<tr>
<td>NC (PBS)</td>
<td>86.0 ± 6.1d</td>
<td>23.7 ± 1.0a</td>
</tr>
</tbody>
</table>

Water (W), hydro-alcoholic (WA) mixture, alcohol (A), a positive control (PC) and a negative control (NC; PBS - phosphate buffered saline) were used. Values are expressed as mean ± SD (n = 3). Values in the same column followed by different letters indicate significant differences (p < 0.05).

3.4.1. Skin compatibility by evaluation of primary skin irritation

Initially, evaluation of irritancy testing was based on visual score. Results obtained by visual analyse are shown in Fig. 4. Results of patch testing demonstrated that hydro-alcoholic provoked a slight erythema in three volunteers 2 h after the patch test removal, however, when statistical analyses were applied regarding negative control, no differences were found. Regarding the positive control, it is possible to observe statistical differences when comparing with negative control and hydro-alcoholic extract (p < 0.05).
investigations revealed that the three extracts of CS validated be
0.05), which indicates the absence of barrier disruption. Con-
compared to the positive control (Fig. 5).
No significant differences were observed for TEWL measure-
ments, in comparison with purified water, 2 h after patch removal (p <
0.05), which indicates the absence of barrier disruption. Con-
sequently, CS hydro-alcoholic extract is not potentially irritant.
The only effect on TEWL values was probably due to the even-
tual formation of a greasy film by the lipophilic components of the extract.
Compared with other extracts that are under investiga-
tion, CS could be consider as safe for cosmetic purposes (Almeida et al., 2008;
Dal’Belo et al., 2006). Unlike arnica and Calendula offic- inalis, CS did not originate skin irritation (Reider et al., 2001). As expected, SLS (PC) leads to an increase of TEWL values.

4. Conclusion
This study constitutes an objective evaluation of the safety of CS extract as cosmetic ingredient and contributes to the elucidation of its mechanism of action. It is an overview of the effect of CS extracts in a validated human skin model for irritancy and eye model under validation. In vitro studies on ocular and dermal irritation were car-
ried out with three different extracts of CS. The in vitro study was carried out with the extract that revealed the best results for the in vitro assays. Results of in vitro studies revealed that CS extracts are safe regarding to skin and ocular irritancy as cell viability was equal to the negative control in both assays and the IL-1α was under 50%. The histological analysis demonstrated that extracts did not affect the skin neither the ocular model. Quantitative chromato-
graphic investigations revealed that the three extracts contained caffeine and HMF, but there were no traces of CGA. The in vitro patch tests proved that the hydro-alcoholic extract did not cause skin irri-
tation. The next steps to evaluate the safety of the extract would undergo sensitization and tolerance studies in normal condition of use.

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