Article

Strategy to Avoid Skin Sensitization: Application to Botanical Cosmetic Ingredients

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Abstract: Assessment of skin sensitization potential is mandatory for ingredients dedicated to topical applications. A battery of in vitro tests covering the key steps of the Adverse Outcome Pathway (AOP) for skin sensitization has been recommended to replace animal testing. However, despite international guidelines on in vitro methods, there is no universal approach defining their combination. The purpose of this work was to assess skin sensitization of botanical ingredients relying on a previously developed in vitro testing strategy. This tool focused on complex and poorly water-soluble substances, which were not already covered. Sixteen botanical extracts were tested in a sequential approach, starting with Sens-Is, supplemented by Organization for Economic Cooperation and Development (OECD) in vitro methods when necessary. The results on the selected well-known botanical sensitizers complemented the relevance of the strategy. Testing on experimental botanicals could detect skin sensitizers. In addition, phytochemistry was a determining support to identify and remove the components at the origin of the effect. Altogether, these results enlarged the scope of the methodology to various ingredient categories and chemical natures, contributing to place on the market new ingredients, safe for workers and end-users.

Keywords: skin sensitization; in vitro; botanical; cosmetic ingredients

1. Introduction

The prevalence of allergic contact dermatitis (ACD) is increasing continuously in Europe and worldwide. A review of 28 studies published between 2007 and 2017 covering 20,107 patch test individuals in the general population found a pooled prevalence of around 20% in Europe, with 16.5% in children and adolescents, 27.9% in women and 13% in men [1]. Dermatologists highlighted that allergen trends have clearly evolved over a defined period of time, sometimes in a short period with the emergence of new allergens [2]. The frequency of occurrence of this skin pathology and the evolving context justify the need to assess skin sensitization potential for any ingredient intended for topical applications. Since the ban on animal testing in Europe, and its extension to many parts of the world, a battery of in vitro tests covering the key steps of the Adverse Outcome Pathway (AOP) for skin sensitization is recommended (OECD Series on Testing and Assessment No. 168 [3]). The AOP concept describes a sequential chain of causally linked Key Events at different levels of biological organization that lead to an adverse health effect. In 2012, a document describing the skin sensitization AOP was published (OECD Series on Testing and Assessment No. 168). Four events are recognized as key, following the prerequisite of skin penetration: (1) molecular interaction with skin proteins; (2) response of activated keratinocytes with inflammatory and gene expression associated with cell signaling pathways (e.g., Antioxidant/electrophile Response Element-dependent pathways—ARE); (3) activation of dendritic cells associated with specific cell surface markers expression, chemokines and cytokines; and (4) T-cell activation and proliferation (Figure 1).
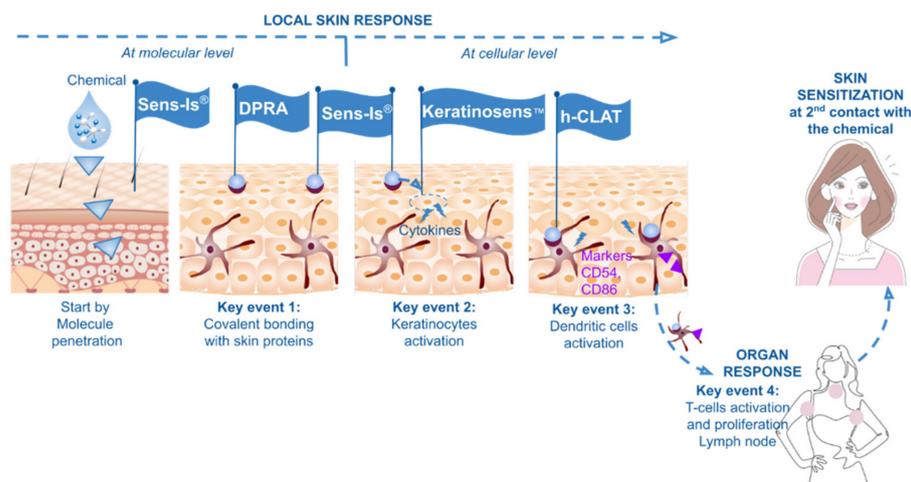
Figure 1. Schematic representation of the sensitizing Adverse Outcome Pathway (AOP). Relationships between the Key Event(s) and the in vitro models.

This concept, combined with international in vitro method guidelines, has been taken up and integrated into a number of guidance documents in order to characterize the sensitizing potential of a substance (e.g., emitted at the worldwide level by OECD; at the European level by ECHA: European Chemicals Agency). To date, three in vitro methods are validated and described in the OECD guidelines (442C, 442D, 442E), and many others are under validation by governmental organizations on alternative test methods. Recently, defined assessment approaches were published (OECD 497 [4]) to predict the skin sensitization hazard, but they were based mainly on the testing of simple and "easy to test" substances. In this context, an internal testing strategy [5] focused on "difficult-to-test" ingredients was developed. "Difficult-to-test" ingredients included poorly water-soluble (<60 mg/L), surfactants and/or complex substances (Unknown or variable composition, complex reaction products or biological materials called UVCB). This ingredient category represents a big part of the cosmetic formulation components. Based on the skin sensitization AOP, the approach consisted in combining complementary in vitro models. The first part of this article will briefly describe this methodology.

Nowadays, sustainability is essential in the creation of a cosmetic product. Although sustainable development can be approached through different perspectives, the naturality of ingredients often plays a central role for consumers. Among plant-based materials, botanical extracts are key for the efficacy of cosmetics. Some of them were reported as potential causes of contact allergy dermatitis [6]. Moreover, botanical extracts are known to be complex and complicated compounds to be tested using in vitro test models.

Considering this perspective, the purpose of this work was to assess the skin sensitization of botanical ingredients with, among them, experimental extracts currently under development.

2. Materials and Methods

2.1. Materials

Two well-known sensitizing botanical extracts: Evernia prunastri absolute and Styrax tonkinensis resin extract were included as positive references [7].

Thirteen examples of botanical extracts, with complex chemical composition, were selected. These examples illustrate the diversity in the design that can be encountered during the development of botanical active ingredients (Table 1). First, the data set included both terrestrial and marine biomass origin from different families. Secondly, diverse preparations were carried out from plant leaves, roots or fruits, mainly by conventional extraction process and, for one of the extracts, by biotechnology. Some botanicals were prepared as dried or oil extracts (100% active substance). Others were prepared in solvents of various polarity (hydrophilic solvents such as glycerin, or glycols, or lipophilic solvents

such as Caprylic/Capric-C8/C10-triglyceride) at lower active substance concentration (i.e., <5%). Thirdly, the ingredients covered the range of solubility from not soluble in water to freely soluble. The final hydrophilic or lipophilic character is an important parameter with regard to the delivery of the active ingredient and the selection of the vehicle (formulation with aqueous or oily continuous phase). The selected botanical extracts were designed to target various biological efficacies (moisturizing, anti-ageing, soothing, skin brightening, skin barrier protection). Regarding expectations of skin sensitization, two experimental extracts, *Hippophae rhamnoides* and *Commiphora myrrha*, were already detected as sensitizers. The skin sensitization profiles of the other eleven botanicals were unknown.

Table 1. Characteristics of the botanical extracts.

Botanical Source	Origin	Preparation	Active Substance (%)	Solubility In Water
Orbignya phalerata (Babassu)	Terrestrial	Dried extract (Amylopectin)	100	Not Soluble
Aphloia theiformis (Vahl)	Terrestrial	Dried extract	100	Poorly soluble
Hippophae rhamnoides (Seabuckthorn)	Terrestrial	Dried extract	100	Soluble
Commiphora myrrha (Myrrh)	Terrestrial	Dried extract	100	Poorly soluble
Apiaceae herb *	Terrestrial	Oil extract (Virgin)	100	Not Soluble
Apiaceae herb *	Terrestrial	Oil extract (Refined)	100	Not Soluble
Ulva lactuca (Sea lettuce)	Marine	Extract in Butylene glycol	<5	Soluble
Beta vulgaris (Beet)	Marine	Extract in C8-C10 Triglyceride	<5	Not Soluble
Hedychium coronarium (Butterfly ginger)	Terrestrial	Extract in Glycerin	<5	Soluble
Helichrysum stoechas (Moench)	Terrestrial	Culture lysate in Glycerin **	<5	Soluble
Asparagopsis armata (Harpoon weed)	Marine	Extract in Propanediol	<5	Soluble
Isatis tinctoria (Woad)	Terrestrial	Extract in Propanediol	<5	Soluble
Arctium lappa (Great burdock)	Terrestrial	Extract in Propylene glycol	<5	Soluble

* The genus and species are subject to confidentiality. ** Biotechnology process.

The research projects on botanicals followed the principles of the Convention on Biodiversity. The samples of natural origin used in this work were accessed and utilized in compliance with the Nagoya Protocol and relevant national laws on accessing genetic resources and sharing the benefits arising from their use. The status of plant and marine resources were constantly monitored with regard to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and to the International Union for Conservation of Nature (IUCN lists).

2.2. Methods

Two methods with OECD guidelines were used: 442D (ARE-Nrf2 Luciferase Test Method- KeratinoSens), 442E (human Cell Line Activation Test h-CLAT) and the Sens-Is assay [8], currently in the work plan of the OECD, chosen for its ability to overcome solubility issues and to discriminate between irritants and sensitizers.

2.2.1. KeratinoSens™

KeratinoSens (Givaudan SA, Switzerland) (Figure 2) is an in vitro assay focusing on the activation of the Keap1-Nrf2-ARE (Kelch-like ECH-associated protein 1—Nuclear factor erythroid—Related Factor 2-Antioxidant Response Element) pathway in transfected keratinocytes containing a luciferase gene, exposed to a test chemical. A concentration series of test chemicals based on the maximum achievable dilution, prepared in either aqueous media or Dimethyl Sulfoxide (DMSO), were applied to the cells for 48 h. Viability was then measured using the MTT test ((3-(four, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide: MTT; 27 μL of a solution at 5 mg/mL in Dulbecco's phosphate-buffered saline was added to each well). Keratinocyte activation was assessed by luciferase measurement after addition of luciferin in the wells (50 μL in each well). Negative control

was defined as the culture medium with or without the solvent used as a vehicle for test item preparation. The positive control was the Cinnamic Aldehyde (CAS No. 14371-10-9) tested at concentrations ranging from 4 to 64 μM. A test chemical was considered positive when the luciferase expression is higher by a factor of at least 1.5 compared to control wells with a dose-effect relationship. Furthermore, the cytotoxicity level must be below 30%.
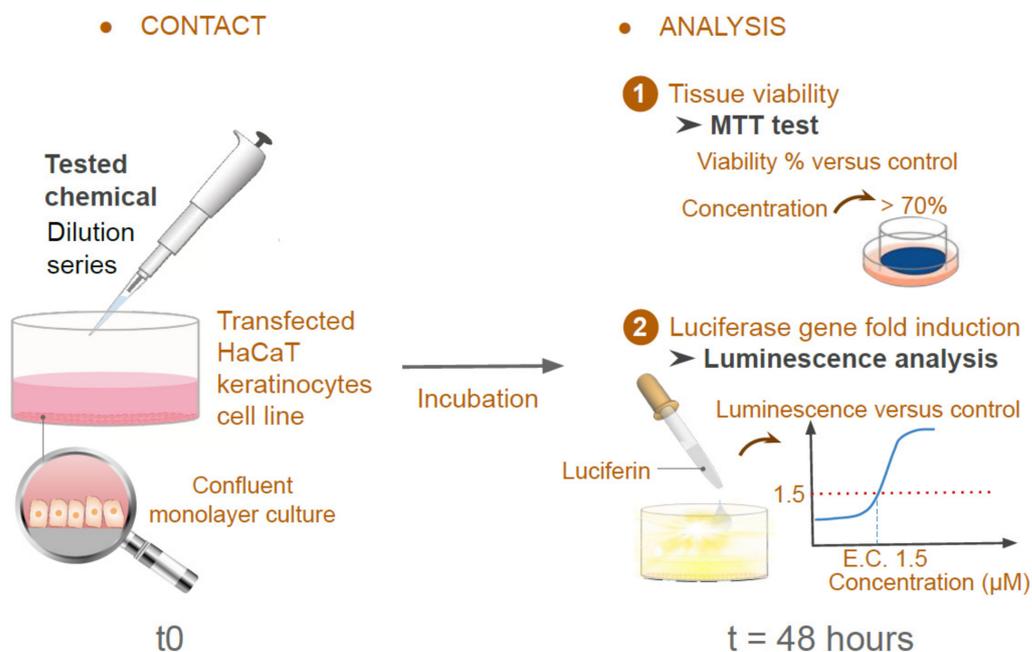
Figure 2. KeratinoSens protocol according to OECD 442C.

2.2.2. Human Cell Line Activation Test (h-CLAT)

h-CLAT (Figure 3) is an in vitro assay quantifying changes in the expression of the CD86 and CD54 (Cluster of Differentiation 86 and 54) membrane phenotypic protein markers in a human monocytic leukemia cell line (THP-1 cells) exposed to a test chemical. Based on the CV75 value (i.e., concentration inducing 25% cytotoxicity), THP-1 cells were incubated with a series of eight concentrations (in either aqueous media or DMSO) for a period of 24 h. Cells were then labeled with fluorochrome-coupled anti-CD86 and anti-CD54 antibodies (50 μL antibody solution; 6μL anti-CD86 in 44 μL staining buffer and 3 μL of anti-CD54 in 47 μL of staining buffer) and analyzed by flow cytometry in order to determine the Relative Fluorescence Intensity (RFI). Propidium iodine (50 μg/mL diluted in PBS) assessed cell viability. Negative control was defined as Lactic acid (CAS No.: 50-21-5) tested at 1000 μg/mL. Positive control was 1-chloro-2,4-dinitrobenzen (DNCB; CAS No.: 97-00-7) tested at 4.0 μg/mL. A test chemical was considered positive as long as the expression of at least one of the two cell surface markers was reproducible and notably increased (i.e., CD86 RFI $\geq$ 150% and/or CD54 RFI $\geq$ 200% in at least one tested concentration). These results should be obtained in two independent runs and in the absence of strong cytotoxicity (i.e., viability $\geq$ 50%).

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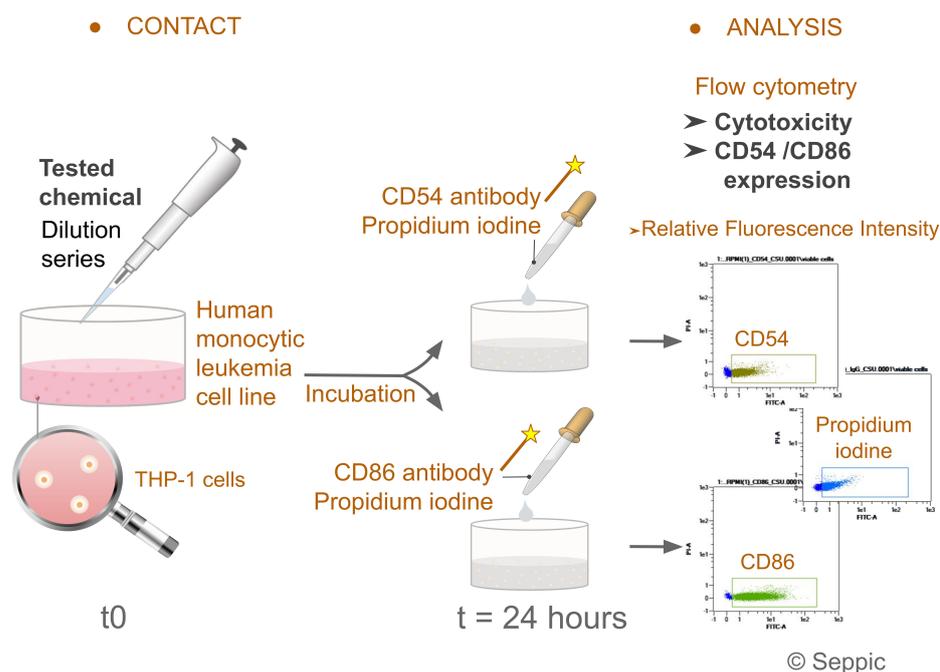
Figure 3. Human Cell Line Activation Test (h-CLAT) protocol according to OECD 442E.

2.2.3. Sens-Is®

Sens-Is (Grasse, France) (Figure 4) is an in vitro assay analyzing the expression of a panel of genes relevant to the biological processes [8] in a reconstructed human epidermis model (Episkin® (Lyon, France)) exposed to a test chemical. Two sets of genes involved in skin sensitization mechanisms are followed; (1) called 'REDOX' focuses on the Keap1-Nrf2-ARE dependent pathway, (2) named 'SENS-IS' is linked to signals leading to the activation of dendritic cells. Chemicals binding to cysteine activate the 'REDOX' group of genes, whereas chemicals binding only to lysine activate genes within the 'SENS-IS' group. The Sens-Is assay discriminates between irritants and sensitizers using a third group of dedicated genes. The experimental protocol follows the Immunosearch Standard Operating Protocol (SOP). Briefly, human 3D reconstructed epidermis were exposed for 15 min to the test chemical (30 µL), pure when possible or dissolved in Phosphate Buffered Saline (PBS), olive oil, DMSO or Dipropylene Glycol. After washing and 6 h post-incubation, tissues were prepared for complementary Deoxyribonucleic Acid (cDNA) quantification by Reverse Transcriptase—Polymerase Chain Reaction (RT-PCR). For each analysis, three negative controls (PBS, olive oil and DMSO), a positive irritation control Sodium Lauryl Sulfate (CAS No. 151-21-3) at 5%, and a positive sensitization control 2,4,6-trinitrobenzene sulfonic acid (TNBS; CAS No. 2508-19-2) at 1%, were performed. A test item was considered a sensitizer when at least seven genes in either group of genes are overexpressed (at least 1.25-fold control value).
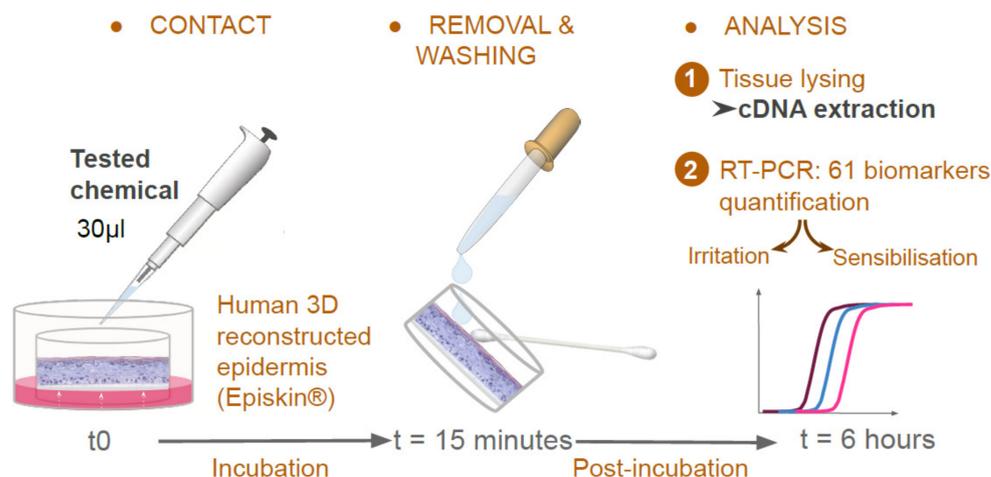
Figure 4. Sens-Is Assay according to Immunosearch Standard Operating Protocol (SOP), analyzing the expression of a panel of genes by RT-PCR in a reconstructed human epidermis model exposed to topical application of a test chemical.

### 3. Description of the Methodology Used to Assess Skin Sensitization

During the methodology development, previously published [5], the results of "difficult to test" ingredients (i.e., poorly water-soluble components, complex substances, etc.), well-known as sensitizers and non-sensitizers, were firstly analyzed on each model. Sens-Is assay showed the highest accuracy and ability to detect true sensitizers. h-CLAT and KeratinoSens showed a lower ability to properly classify potential sensitizers. Finally, Direct Peptide Reactivity Assay (DPRA; OECD 442C), initially included in the experimental models, appeared unsuitable. The statistical analysis of the results highlighted the need to combine different methodologies to increase the reliability of the prediction.

The Sens-Is model was chosen as a pivotal test for additional benefits [9,10]:

- Possibility of analyzing any substance whatever its solubility, physical state, or surfactants that could induce cytotoxicity on 2D cell culture models;
- Ability to discriminate between irritants and sensitizers;
- Proper prediction of the pre- or pro-haptens [11];
- Evaluation of the two first Key Events of the skin sensitization AOP and taking the dermal penetration of the compounds into account.

KeratinoSens enabled assessment of Key Event 2 in a complementary way of cell exposure. h-CLAT was an essential validated method to evaluate Key Event 3.

A sequential strategy was defined (Figure 5): Sens-Is was the entry test, followed by h-CLAT and KeratinoSens. In a conservative approach, a Sens-Is positive result was considered sufficient to classify the tested ingredient as a skin sensitizer and implied that no other test needs to be performed. In the case of a negative result, this figure must be confirmed by another in vitro test, the h-CLAT. Hence, two concordant results covering the first three KE of the AOP allowed us to conclude on the absence of sensitizing potential. In the case of non-concordant results, a third test (KeratinoSens) was necessary to conclude.
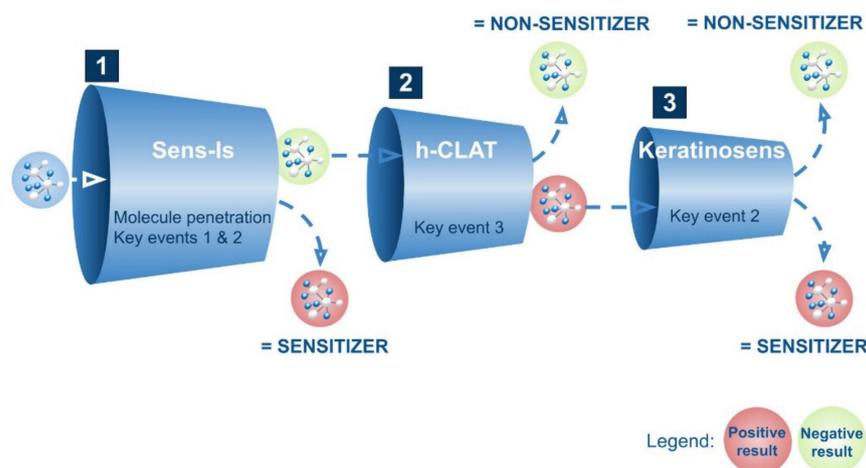
Figure 5. Decision tree to assess skin sensitization hazard. Sens-Is assay was used as a starting test, followed by the h-CLAT if negative outcome. KeratinoSens was used to conclude when a discrepancy occurred between the results of the two first models. In case of a positive result with Sens-Is, the chemical was concluded as a skin sensitizer.

Statistical analysis of the results compared to in vivo data showed an accuracy of 96% (24/25), with a sensitivity of 100% (11/11) and a specificity of 93% (13/14). These percentages were higher than 80% and higher than the individual tests were. The testing strategy was able to minimize the risk of a false negative conclusion.

4. Results of Botanical Ingredients

Results obtained with experimental models are presented in Table 2. Well-known skin sensitizers, *Styrax tonkinensis* resin extract, *Evernia prunastri* absolute, provided a positive response in Sens-Is, leading to their categorization as sensitizers according to the testing strategy.

Table 2. In vitro results analysis and sensitization potential assessment.

Botanical Extract	Results on Each in vitro Model			Sensitization Potential
	Sens-Is	h-CLAT	Keratino-Sens	
Evernia prunastri absolute	Sensitizer	NT	NT	Sensitizer
Styrax tonkinensis resin extract	Sensitizer	NT	NT	Sensitizer
Orbignya phalerata (Babassu-Amylopectin) extract	Sensitizer	NT	NT	Sensitizer
Aphloia theiformis (Vahl) extract	Non-sensitizer	Non-sensitizer	NT	Non-sensitizer
Hippophae rhamnoides (Seabuckthorn) extract	Non-sensitizer	Non-sensitizer	NT	Non-sensitizer
Commiphora myrrha (Myrrh) extract	Non-sensitizer	NT	NT	Non-sensitizer
Apiaceae herb extract (Virgin)	Sensitizer	NT	NT	Sensitizer
Apiaceae herb extract (Refined)	Non-sensitizer	Sensitizer	Sensitizer	Non-sensitizer
Ulva lactuca (Sea lettuce) extract	Non-sensitizer	Non-sensitizer	NT	Non-sensitizer
Beta vulgaris (Beet) extract	Non-sensitizer	NT	NT	Non-sensitizer
Hedychium coronarium (Butterfly ginger) extract	Non-sensitizer	NT	NT	Non-sensitizer
Helichrysum stoechas (Moench) extract	Non-sensitizer	NT	NT	Non-sensitizer
Asparagopsis armata (Harpoon weed) extract	Non-sensitizer	NT	NT	Non-sensitizer
Isatis tinctoria (Woad) extract	Non-sensitizer	NT	NT	Non-sensitizer
Arctium lappa (Great burdock) extract	Sensitizer	NT	NT	Sensitizer

NT: Not Tested.

For weakly concentrated plant extracts (≤5% Active Substance) and in the absence of a phytochemical alert, only Sens-Is was performed. All of these Sens-Is tests were found to be negative, allowing further confirmation of good tolerance by clinical trials.

The other candidates in concentrated form came from current ingredient development projects for which the strategy was used to assess their skin sensitizing potential. Among

them, *Hippophae rhamnoides*, *Commiphora myrrha* crude extracts, and *Isatis tinctoria* extract in propanediol provided positive Sens-Is responses leading to the conclusion that they are skin sensitizers. Virgin experimental plant oil showed a negative response with Sens-Is assay, while it induced the activation of dendritic cells in the h-CLAT assay. Keratinocyte response was also identified with the KeratinoSens, leading to the conclusion of sensitization potential. The discrepancy in the response could be explained by the model's design. The h-CLAT and KeratinoSens can detect the presence of a sensitizer at very low concentrations. On the contrary, the Sens-Is, similarly to the LLNA, gives a positive response when the concentration of a sensitizing molecule is over the Effective Concentration for a stimulation index of 3 (EC3) limit. *Orbignya phalerata* and *Aphloia theiformis* extract did not induce a positive response in Sens-Is nor in h-CLAT assays and were therefore considered non-sensitizers.

5. Discussion

The in vitro results on the two references, *Styrax tonkinensis resin extract* and *Evernia prunastri* absolute (Table 3), were in line with their well-known hazard classification for skin sensitization. *Hippophae rhamnoides* and *Commiphora myrrha* crude extracts, also identified as skin sensitizers with in vivo studies (unpublished data [12,13]), were properly detected in vitro. Moreover, recent literature on *Commiphora myrrha* [14] reported the identification of two reactive compounds (6-oxofuranodienones and methoxyfuranogermacrenones) susceptible to trigger the molecular initiating event of skin sensitization. These four results completed the statistical analysis of the testing strategy, resulting in an accuracy of 97% (28/29), a sensitivity of 100% (15/15) and a specificity of 93% (13/14). The other twelve botanicals, evaluated during the development of new ingredients, were therefore not considered for statistics, as their sensitizing profiles were unknown at the beginning of this work.

Table 3. In vitro results analysis and sensitization potential assessment.

Botanical Extract	in vitro Evaluation	in vivo Evaluation
Evernia prunastri absolute	Sensitizer	Sensitizer
Styrax tonkinensis resin extract	Sensitizer	Sensitizer
Orbignya phalerata (Babassu-Amylopectin) extract	Non-sensitizer	Non-sensitizing [a] (10%)
Aphloia theiformis (Vahl) extract	Non-sensitizer	Non-sensitizing [a] (0.01%)
Hippophae rhamnoides (Seabuckthorn) extract	Sensitizer	Sensitizer [b]
Commiphora myrrha (Myrrh) extract	Sensitizer	Sensitizer [c]
Apiaceae herb extract (Virgin)	Sensitizer	NT
Apiaceae herb extract (Refined)	Non-sensitizer	NT
Ulva lactuca (Sea lettuce) extract	Non-sensitizer	Non-sensitizing [a] (8%)
Beta vulgaris (Beet) extract	Non-sensitizer	Non-sensitizing [a] (Pure)
Hedychium coronarium (Butterfly ginger) extract	Non-sensitizer	Non-sensitizing [a] (4%)
Helichrysum stoechas (Moench) extract	Non-sensitizer	Non-sensitizing [a] (1%)
Asparagopsis armata (Harpoon weed) extract	Non-sensitizer	Non-sensitizing [a] (3%)
Isatis tinctoria (Woad) extract	Sensitizer	Structure alert
Arctium lappa (Great burdock) extract	Non-sensitizer	Non-sensitizing [a] (1%)

[a] [15]; [b] [12]; [c] [13]; NT: Not Tested.

Isatis tinctoria extract was detected as a sensitizer. A deeper phytochemical analysis revealed traces of suspected sensitizing compounds, which strengthened confidence in the result. One of the compounds, isatin [16], was specifically questioned.

Following the identification of virgin plant oil sensitization potential, further phytochemical composition was investigated. CPG analysis highlighted the presence of residual volatile organic components (VOC) with more than 30 terpene molecules, representing 3.3% in the oil composition. Among them, linalool was quantified as the main compound (i.e., around 1.4% of the total virgin oil) and was suspected to be responsible for the effect

observed. Therefore, a purification step, dedicated to reduce VOCs, was added to the manufacturing process. The refined plant oil showed a residual VOC content of 30 ppm, in which one third is composed of linalool (i.e., 10 ppm). The absence of sensitization potential of this refined oil supported the previous hypothesis and made it possible to confirm the local tolerance at a clinical level.

Other botanical extracts with in vitro non-sensitizing responses, *Orbignya phalerata*, *Aphloia theiformis*, *Ulva lactuca*, *Beta vulgaris*, *Hedychium coronarium*, *Asparagopsis armata*, *Arctium lappa*, and *Helichrysum stoechas* culture lysate consistently demonstrated the absence of any allergic reaction during clinical trials at use levels.

6. Conclusions

The in vitro testing strategy previously developed for "difficult to test" ingredients was appropriate for the tested botanical ingredients. It is important to remember that some uncertainties still exist, mostly linked to the limits of the in vitro tests.

The high accuracy of 97% combined with 100% sensitivity removed the risk of false negative conclusions. Implementation of this approach in the development process of new ingredients guarantees the absence of skin sensitization hazards with high confidence in view of clinical steps. The essential contribution of phytochemistry investigations was highlighted for the development of non-sensitizing ingredients. Bearing in mind the strong consumer demand for natural products, the unexpected effects observed with some botanicals reinforces the need for an accurate safety assessment.

The development and availability of new approach methodologies (NAMs) could participate in determining the skin sensitization categories according to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

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