

Application of the KeratinoSens Assay for Prediction of Dermal Sensitization Hazard for Botanical Cosmetic Ingredients

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ABSTRACT

An essential step in the safety review of cosmetic/personal care ingredients is hazard assessment for a series of endpoints, including dermal sensitization potential. In vitro methods have been developed to identify allergic (haptenic) potential for individual chemicals based on electrophilic interaction with marker peptides or cellular target systems. These assays generally use a specific molar ratio of the test chemical to the test system. Botanical extracts are used increasingly in formulas and, as mixtures, specific molar ratios cannot be determined for these assays. Often, the botanical extract portion is a relatively small portion of the complete ingredient. To assess these mixtures, the KeratinoSens assay was selected because it operates over a wide dose range and sets cytotoxicity limits on doses used to measure marker gene expression (Emter et al, 2010 [7]). In the KeratinoSens assay, the induction of a luciferase gene, under the control of the antioxidant response element (ARE) derived from the human gene AKR1C2 gene, is measured. In parallel, cytotoxicity is assessed by both Neutral Red Uptake (NRU) and MTT assays. Test concentrations ranged up to 1000 µg/mL (of complete ingredient) and a test concentration was considered positive if the relative viability was $\geq 70\%$ and the fold induction of luciferase was 1.5x relative to the solvent controls. The goal of the study was to measure the activity of 3 known sensitizers (gluteraldehyde (GA) [strong], dimethyl maleate (DM) [moderate] and cinnamic aldehyde (CA) [moderate] spiked into four different botanical ingredients (each with a different excipient solvent systems). The “spiked” botanical ingredients were used as the test article as no sensitizing botanical ingredient was available. Activity of the spiked sample was measured relative to the EC1.5 of the neat sensitizer as a function of sensitizer concentration and extract composition. Three independent trials were performed on each test material. No appreciable cytotoxicity was observed with any of the samples. The recovery of the GA spike required at least a ~ 3 fold increase in concentration relative to the chemical alone and botanical ingredient #3 reduced the activity below detection. The DM and CA showed activity at about the same effective concentrations as the neat chemical although the DM showed reduced activity in botanical ingredient #3 as well. These data suggest that the KeratinoSens assay has the potential to identify electrophile allergens within a botanical ingredient matrix.

INTRODUCTION

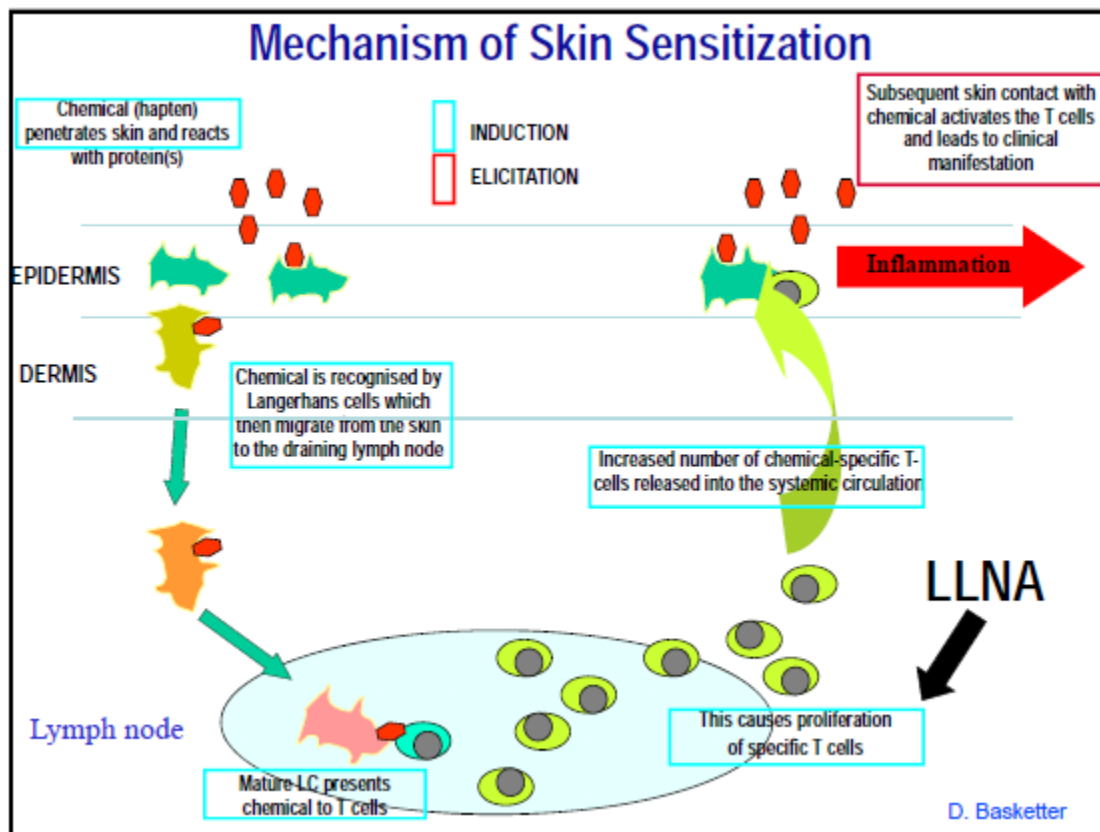
In reviewing ingredients and formulas for use in cosmetic and personal care products, one of the key toxicological endpoints is the potential to induce skin sensitization. Since most of the chemicals used in these ingredients are relatively small molecules (in contrast to proteins in foods), the sensitization of most concern is Type IV, delayed contact hypersensitivity. The process by which a small molecule induces sensitization in the skin is multi-stepped and now well characterized [1, 2]. These molecules are too small to act as immunizing antigens themselves but rather bind to self-proteins in the skin. This is the classic haptenic reaction described decades ago by Landsteiner [3]. The steps in the sensitization process, from skin penetration through to clonal amplification of the specific t-cell clones, are summarized in Figure 1. Measuring the ability of a chemical to act at each one of these steps has provided means to predict its propensity to act as a sensitizer. The Local Lymph Node Assay [2] measures the final step of clonal proliferation in vivo while several in vitro assays measure action upstream of this final step. In vitro skin penetration provides data on chemical availability. The Direct Peptide Reactivity Assay (and related methods) measures the ability of the chemical to act as an electrophile binding to lysine and/or cysteine moieties [4]. Cell-based assays measure upregulation of certain gene products associated with dendritic cell activation. The Myeloid U-937 Skin Sensitization Test (MUSST) [5] measures CD86 expression, and the human Cell Line Activation Test (h-CLAT) [6] measures CD86/CD54 surface expression. The KeratinoSens assay [7], based on the HaCaT cell line, uses a luciferase reporter gene linked to the antioxidant-response-element (ARE). Electrophilic chemicals are detected by inducing conformational changes in the Keap1 target protein, which in turn activates the ARE and leads to an upregulation of the luciferase reporter. The degree of upregulation is measured by relative luminescence and related to cell viability (MTT or NRU). These in vitro test methods have been initially designed to accommodate specific molar ratios of test article to test system or molar limits for cell treatment.

Increasingly, toxicologists are faced with mixtures of ingredients, particularly when natural products are concerned. Botanical extracts are now common components in many cosmetic and personal care products. Performing preclinical sensitization potential evaluation is challenging as molar concentrations of certain components are not known. For example, cosmetic ingredients containing botanical extracts are formulated with solvents, preservatives and generally less than 10% extract. The solvents and preservatives can be characterized as individual chemicals but the botanical extract must be tested as part of the complete ingredient (as delivered by the manufacturer). Thus, an assay and treatment conditions were needed to evaluate the sensitizer as a small fraction of the whole ingredient. For this study, we selected the KeratinoSens assay because of its mode of action and the ability to limit exposures based on

cytotoxicity measurements. In that way, the test article exposure could be maximized without the test system being overwhelmed.

Four botanical extract-containing ingredients were selected to represent different solvent and preservative systems. For the purposes of this study, these were considered different matrices into which the known sensitizers were spiked. Each matrix (complete ingredient) was spiked with known sensitizers at between 1% and 2% of the final volume. These concentrations were selected so that the EC 1.5 (concentration inducing a 50% increase in luciferase signal) would fall within the dose range of the test material (spiked botanical extract ingredient) and the spike concentrations would reflect potential concentrations of the botanical extracts in the complete botanical extract-containing cosmetic ingredient. Two goals were set: 1) could the spiked sensitizers induce an EC 1.5 within the test material dose range selected (up to 1000 µg/mL) and 2) how would the EC 1.5 be impacted by the different botanical extracts?

Figure 1. Diagram of the steps of Induction and Elicitation phases of delayed contact hypersensitivity



MATERIALS AND METHODS

The known sensitizer chemicals (Table 1) were selected to cover the potency range of strong to moderate activity. Four botanical extract-containing ingredients were selected to represent a range of solvent systems generally found in extracts used in cosmetic formulas (Table 2).

Table 1. Sensitizers selected for this study

Name	Supplier	MW	EC 1.5 (μ m)	Sensitization Class	Mode of Action	EC 1.5 Data Source
Gluteraldehyde	Sigma Aldrich	100	20	Strong	Shiff Base	Natsch 2008 [9]
Dimethyl Maleate	Aldrich	172	4.93	Moderate	Michael Acceptor	IIVS
Cinnamic Aldehyde	Aldrich	132	~15	Moderate	Michael Acceptor	IIVS

Table 2. Breakdown of the components in Botanical Ingredients selected

Botanical Ingredient	Matrix 1	Matrix 2	Matrix 3	Matrix 4
Botanical extract	10%	1%	10%	1%
Water	7.5%	49%	40%	49.5%
1,3 butylene glycol	51%			49.5%
Propandiol			50%	
Glycerin		49%		
PEG-8	30%			
preservative	0.5%	0.75%		
other	0.45%	0.25%		

Because the majority of the test substance was solvent rather than a potential sensitizer of interest, the maximum concentration of test substance was increased to 1000 μ g/mL from the standard protocol value of 1000 μ M.

The KeratinoSens Assay was carried out according to methods outlined in Emter *et al.* [7]. Stably transfected HaCaT cells with a 56-base-pair insertion containing the ARE sequence from the AKR1C2 gene upstream of a luciferase gene were obtained from Givaudan. The cells were grown for 24 hours in 96-well plates. The medium was then replaced with medium containing the test substance (dilutions prepared in DMSO) so that the final DMSO concentration in the wells was 1%. Each test substance was tested at 12 dilutions. Each 96-well test plate contained serially diluted test substances, 6 wells with the solvent control (DMSO), 5 wells with the positive control (cinnamic aldehyde) at five different concentrations, and 1 well with no cells for background subtraction. In each assay, three parallel replicate plates were run with this same set-up and a fourth and fifth parallel plate was prepared for cytotoxicity determination. Cells were incubated for 48 hours with the test articles, and then luciferase activity and cytotoxicity (NRU and MTT viability assays) were determined.

The experimental design of this study consists of three assays (1 dose range finding assay and 2 definitive assays) to determine the EC 1.5 value (concentration for induction above 1.5 threshold), and a mean IC₅₀ (concentration resulting in 50% cytotoxicity) for each test article. A dose range finding assay was performed to determine the appropriate test article dilutions for the subsequent definitive assays.

According to the current prediction model when testing on a w/v basis, a test article was predicted to have sensitization potential if: 1) The EC1.5 value fell below 200 µg/mL; 2) at the lowest concentration with a gene induction above 1.5, cellular viability was greater than 70%; and 3) there was an apparent overall dose response which was similar between the definitive assay trials. **However, for the purposes of this research study, each test substance that resulted in an EC1.5 value at any tested concentration was indicated as a potential sensitizer.**

RESULTS

The project was divided into two parts. The first experiments examined three sensitizers spiked into botanical matrix 1 and the second experiments used two of the sensitizers spiked into each of three botanical matrices (Table 3). The data are expressed first as the EC 1.5 of the spiked botanical mixture (in µg/mL) and then as the EC 1.5 of the sensitizer spike in the mixture (µM) and compared to the reference EC 1.5 (µM). The five figures (2a-2e) are taken from the definitive studies with the gluteraldehyde spike and show the relative cell viability (using the MTT endpoint) compared to the relative induction of luciferase (gene expression). The lines are labeled with the laboratory accession codes for each sample.

Table 3. Summary table of the results

Spike	Spike Concentration	EC 1.5 µg/mL (test material)*	IC ₅₀ µg/mL [^] (MTT)	IC ₅₀ µg/mL [^] (NRU)	EC 1.5 (µM) Observed#	EC 1.5 (µM) published
Experiment 1	Matrix 1					
Gluteraldehyde	2%	308.70	>1000	>1000	61.7	20.3
Dimethyl Maleate	2%	38.00	>1000	>1000	4.4	4.93
Dimethyl Maleate	1%	120.10	>400	>400	7.0	4.93
Cinnamic Aldehyde	2%	19.24	>400	>400	7.5	10.2
Cinnamic Aldehyde	1%	120.40	>400	>400	9.1	10.2
Cinnamic Aldehyde	(Pos. Control)				10.2	NA
Experiment 2						
Matrix 2	2% Gluteraldehyde	356.9	>1000	>1000	71.4	20.3
Matrix 3	2% Gluteraldehyde	>1000	>1000	>1000	>200	20.3
Matrix 4	2% Gluteraldehyde	501.5	>1000	>1000	100.3	20.3
Matrix 2	1% Dimethyl Maleate	133.6	>1000	>1000	7.8	4.93
Matrix 3	1% Dimethyl Maleate	467.6	>1000	>1000	27.2	4.93
Matrix 4	1% Dimethyl Maleate	203.5	>1000	>1000	11.8	4.93
Cinnamic Aldehyde	(Pos. Control)				13.3	

NOTE: When an EC1.5 value or IC50 value was not obtained, the results are presented as greater than the highest dose tested.

* The EC1.5 value is the concentration for gene induction above the threshold (1.5 fold) as compared to the DMSO solvent controls. These values are the average of the values from the two definitive trials for each test material.

[^] The IC50 value is the concentration at which cells are 50% viable as compared to the DMSO solvent control wells. Cell viability was measured using two separate cytotoxicity assays (MTT and NRU).

The EC1.5 value observed (in µM of the sensitizer) was determined by calculating the concentration of the sensitizer spike in the EC 1.5 of the test material and converting that value to µM.

Figure 2a Botanical Matrix #2 without any spike (MTT viability)

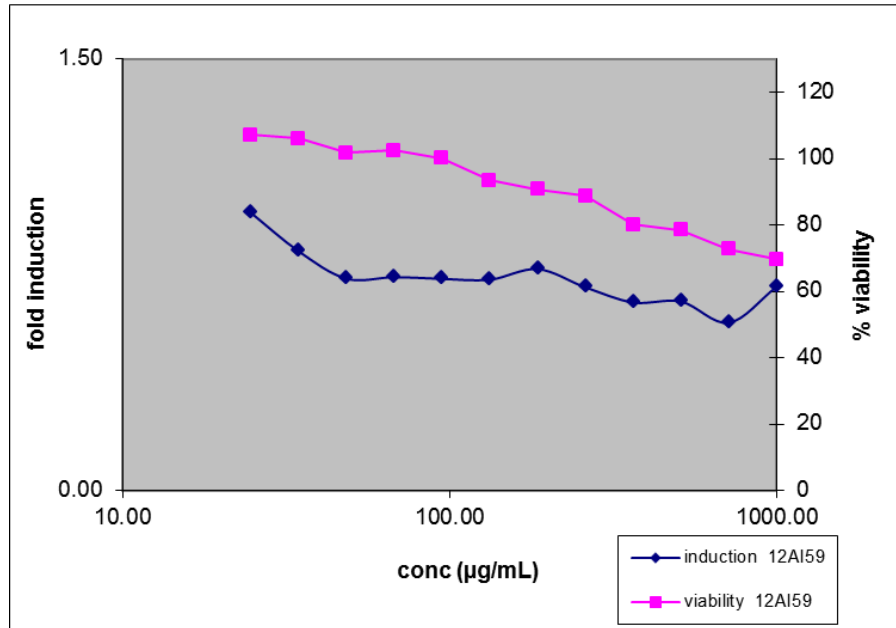


Figure 2b Botanical Matrix 1-Glutaraldehyde 2% (MTT)

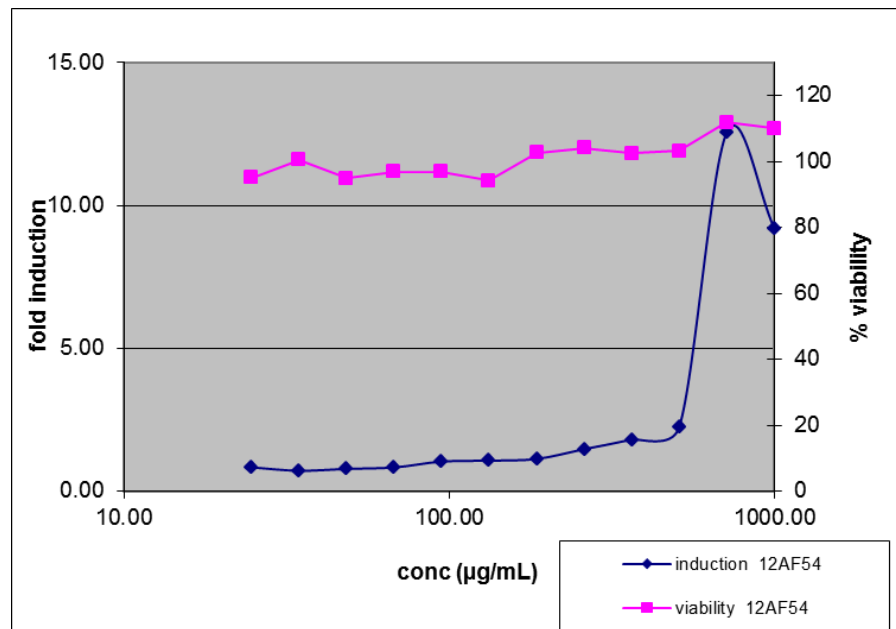


Figure 2c Botanical Matrix 2 –Gluteraldehyde 2% (MTT)

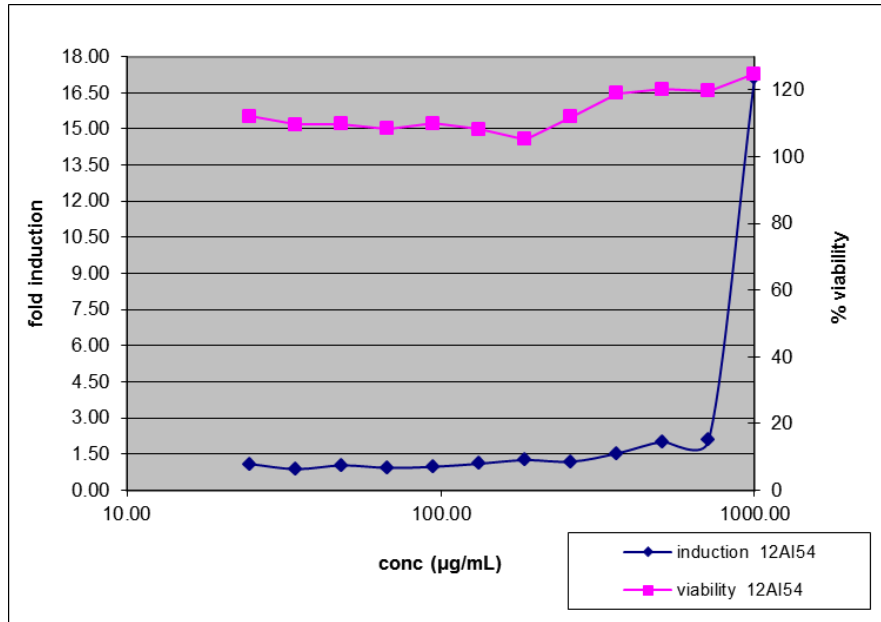


Figure 2d Botanical Matrix 3-Gluteraldehyde 2% (MTT)

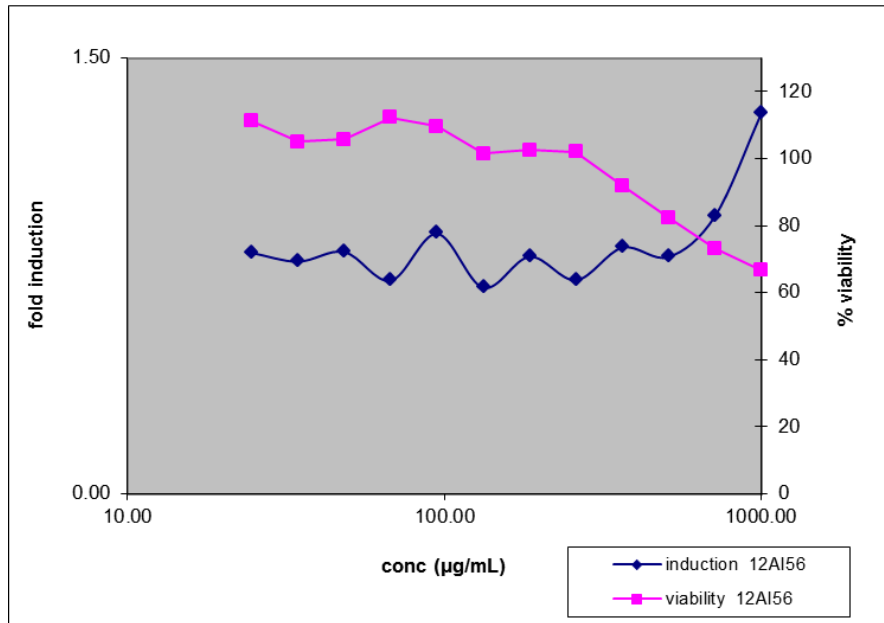
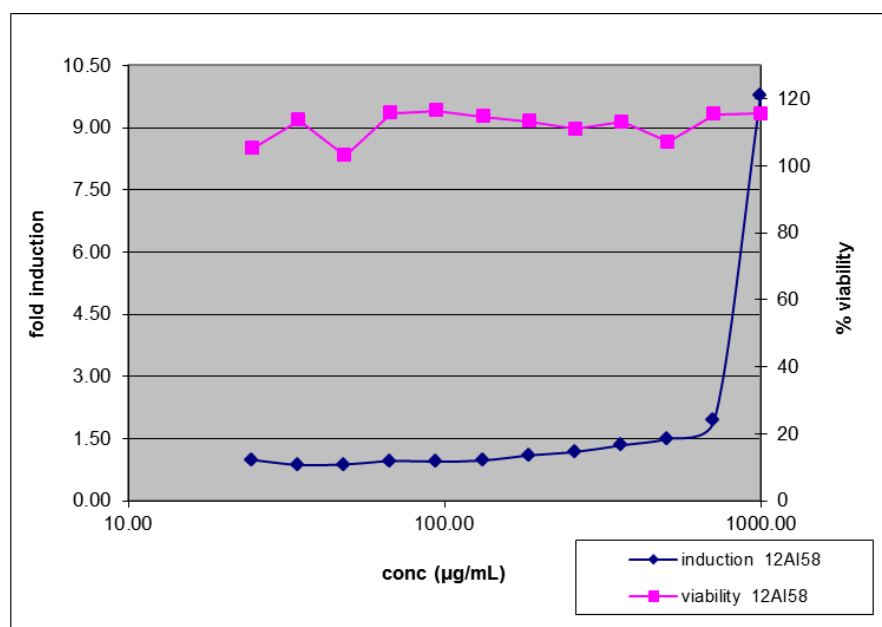


Figure 2e Botanical Matrix 4-Gluterldehyde 2% (MTT)



DISCUSSION

The goal of preclinical testing is to identify potential sensitizing ingredients (especially strong to moderate sensitizers) so that they do not progress into clinical testing. KeratinoSens, and assays like it, are intended to predict hazard. They rely on assured exposure of the test material to the test system and quantitative measurement of the specific assay endpoint(s). Considerations of risk, such as skin penetration of the test chemical and enhanced/diminished penetration effects from the vehicle(s), are not a focus at this stage of testing. Because mixtures, including botanical extracts, are not amenable to quantitative molar exposures to the test systems, a test system was needed that would accommodate indeterminate molar ratios while still limiting exposures so as not to overwhelm the test system. The KeratinoSens assay uses a wide treatment concentration range and limits the acceptable degree of relative cytotoxicity thus ensuring a biologically relevant outcome (hazard assessment).

The goals of this initial study were two-fold:

- To determine the ability to recover activity from modest concentrations of known strong and moderate sensitizing chemicals when spiked into commercial botanical extract-containing mixtures commonly used as cosmetic ingredients.
- To measure the impact (shift) in EC 1.5 values as a function of the different ingredient systems used. This goal was not intended to differentiate between the impacts of the botanical itself and the solvent system used in the complete ingredient.

Both goals were met for this limited data set. In the first experiment, sensitizer spikes at 1 to 2% were sufficient to induce a positive upregulation of the ARE below the maximum test material concentration of up to 1000 µg/mL. These responses occurred at cell survivals well in excess of 70%. The botanical matrix did impact recovery for both the gluteraldehyde and dimethyl maleate sensitizers.

These initial data suggest that the KeratinoSens assay may be used for the initial assessment of botanical ingredients. Positive responses would suggest that the mixture has sensitization potential and is an unlikely candidate for clinical confirmation. A negative response would be part of an overall weight of evidence assessment to support confirmation of the absence of sensitization potential in the clinic.

CONCLUSIONS

The two goals of the study were met for this limited data set:

- The sensitizer spike could be recovered from the botanical matrix in the form of an EC 1.5 within the limits of exposure concentration and cell viability.
- The botanical matrix did impact the recovery of each sensitizer spike. Shifts in the EC 1.5 for gluteraldehyde ranged from 3 fold to >10 fold compared to gluteraldehyde alone while the shift for dimethyl maleate ranged from none to ~ 5 fold. There are several possible explanations for the decreased activity. Among these is the role of amines or other nucleophile within the botanical extract that could compete for the electrophilic sensitizer binding.
- From the EC 1.5 of the mixture, it may be possible to estimate a relative potency for the mixture but not one for the active component(s).

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