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The sensitivity of the KeratinoSens™ assay to evaluate plant extracts: A pilot study

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ABSTRACT

Several tests to assess skin sensitization hazard are in peer-review for pre-validation. These tests, as well as the animal tests they aim to replace, were developed (and validated) for the testing of pure substances. However, in the cosmetic field, active ingredients are often mixtures from natural sources. It is therefore important to understand which tests could be used to evaluate their safety. Here we describe a proof-of-concept study to test whether the KeratinoSens™ assay is able to detect sensitizing constituents within botanical mixtures. Four extracts were spiked with different doses of the sensitizers citral, cinnamic aldehyde and isoeugenol. The tested extracts were negative in the test whereas they became positive in most cases when spiked with the sensitizers. Analysis of the results from the samples spiked with different doses allowed the determination of the minimal level of sensitizers being detectable. The contribution to sensitization potential of doses of 2% and above of the spiked sensitizers were reliably detected. There were limitations for an extract with high cytotoxicity, in which case detection of the artificially spiked sensitizers proved difficult. This study gives a proof of principle for testing of mixtures in the KeratinoSens™ assay and indicates how sensitive the assay is to detect minor components with sensitizing potential.

1. Introduction

Cosmetic legislation in Europe has imposed a ban on animal testing for the evaluation of skin sensitization of cosmetic ingredients by 2013. Therefore, predictive *in vitro* tests are being developed and several tests are in the process of pre-validation. At the same time, the REACH regulation (Registration, Evaluation, Authorization and Restriction of Chemicals) (REACH, 2007) requires the testing of thousands of not previously tested substances for their skin sensitization potential, which will increase the numbers of animal tests needed (Rovida and Hartung, 2009). Currently, the skin sensitization potential is estimated with the local lymph node assay in mice (LLNA) (Basketter et al., 2002). The result of the LLNA is reported as the EC3 indicating the estimated concentration of a chemical required to produce a 3-fold stimulation of draining lymph node cell proliferation in the LLNA assay compared with concurrent solvent controls.

REACH requires not only pure substances to be registered, but also requires registration of mixtures extracted from natural sources. However, classical animal tests such as the LLNA normally were only validated against pure preparations and there is no validation for and little experience with testing of mixtures. The LLNA was nevertheless used to test a number of essential oils (Lalko and Api, 2006). Oils with high levels of known moderate sensitizers such as the citral-containing *Litsea cubeba* and lemongrass oils or the eugenol-containing clove oil were equally positive in the LLNA as the pure substances were. Essential oils containing only a fraction of weak sensitizers such as geranium oil and citronella oil were negative in this study.

In the cosmetic field, there is an increasing interest in using naturally derived complex botanical mixtures as active principles. Both for regulatory purposes and to provide an adequate risk assessment by the producer of the finished product, an indication of the potential sensitization risk stemming from these botanical mixtures is needed. Whilst there is little experience to perform this risk assessment on mixtures with animal tests, there is even less experience whether the novel *in vitro* tests could be applied to inform such a risk assessment.

Abbreviations: LLNA, local lymph node assay; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; SOP, standard operating procedure; ECVAM, European centre for the validation of alternative methods to animal testing; DMSO, dimethylsulfoxide; IC50, inhibitory concentration for 50% reduction in viability as determined with the MTT assay; EC 1.5, extrapolated concentration for 1.5-fold luciferase induction above threshold; REACH, Registration, Evaluation, Authorization and Restriction of Chemicals.

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Table 1
Plant extracts evaluated in this study.

Origin	Solid/active plant matter content	Vehicle/solvent of the extract
Green tea extract	100%, (mainly polyphenols)	Pure, no vehicle
Matricaria chamomilla Linné (chamomile)	20%	27% propyleneglycol, 53% H_2O
Carica Papaya (Papaya) fruit	0.7%	Glycerol
Paullinia cupana (Guarana) seed	0.7%	Glycerol

The KeratinoSens™ assay is a reporter cell-based approach based on the finding that the majority of the skin sensitizers induce the Nrf2-Keap1-ARE regulatory pathway (Ade et al., 2009; Natsch, 2010; Natsch and Emter, 2008; Vandebriel et al., 2010). The antioxidant response element (ARE) from the human AKR1C2 gene was inserted in front of a SV40 promoter and placed upstream of a luciferase gene. Stable insertion of the resulting construct in HaCaT keratinocytes resulted in the KeratinoSens™ reporter cell line. Induction of luciferase in this cell line can thus be used to screen for skin sensitizers. Here, we report results from a pilot study using the KeratinoSens™ assay to compare different plant extracts which have artificially been spiked with different doses of the naturally occuring moderate dermal sensitizers citral, cinnamic aldehyde and isoeugenol. By spiking the extracts, the ability and sensitivity of the assay to detect such moderate (or stronger) potential sensitizing components was explored.

2. Materials and methods

2.1. Botanical extracts studied and spiking of the extracts

Table 1 summarizes the plant extracts studied. Green tea leaf solid extract (*Camelia Sinensis* Extract (and) Silica (EU 2006/257/EC), INCI 9419, CAS: 84650-60-2) was obtained from Cognis, Dusseldorf, Germany. This brown powder is mainly composed of polyphenols (95%), with high catechin content (50% related to the dry substance).

Chamomile flower glycolic extract (*Matricaria chamomilla* Linné) was obtained from Grupo Centroflora, Botucatu SP, Brazil. This brown liquid contains 20% of active plant matter content, solubilised in 27% propylenglycol and 53% water. The extract contains flavonoids as the main active ingredients.

Papaya fruit extract (*Carica Papaya*) and Guarana seed extract (*Paullinia cupana*) were obtained from Alban Muller international, Vincennes, France. These extracts are standardized extracts at 0.7% of active plant content, solubilised in water (2.5%) with glycerol as carrier (96.8%).

The spiking agents citral, cinnamic aldehyde and isoeugenol were directly added to the liquid extracts at the indicated doses, and the spiked extracts were then used for the test. In the case of the green tea extract, which is a powder, this was not considered adequate as a homogeneous mixture would not be achieved. In this case therefore both the extract and the spiking agent were separately added to the DMSO solvent used for testing.

2.2. Test procedure and standard operating procedure (SOP) of the KeratinoSens™ assay

The KeratinoSens™ cell line has been described in detail (Emter et al., 2010). All tests were run according to the previously published SOP (Emter et al., 2010). Cells were grown for 24 h in 96-well plates. The medium was then replaced with medium containing the test substance and a final level of 1% of the solvent, DMSO. Each test preparation was tested at 12 two-fold dilutions. According to the SOP, pure preparations of chemicals with no defined molecular weight are tested in the range from 0.2 to 400 ppm. Since the test items contained themselves a significant amount of vehicles, the maximal test concentration was increased to 1000 ppm or, for the two most dilute extracts (Guarana seed and Papaya fruit), to 10,000 ppm. In each repetition, three parallel replicate plates were run for luciferase determination and a fourth parallel plate was prepared for cytotoxicity determination. Cells were incubated for 48 h with the test substances, and then luciferase activity and cytotoxicity (with the MTT assay) were determined. This full procedure was repeated three times for each test preparation, thus generating nine luciferase induction data points and three MTT data points for each test item at each concentration.

2.3. Data analysis and statistical evaluation

For each test item in each repetition and at each concentration, the gene induction compared to DMSO controls and the wells with statistically significant induction over the threshold of 1.5 (i.e. 50% enhanced gene activity) were determined. Furthermore the maximal fold-induction (I_{max}) and the EC 1.5 value (concentration in ppm for induction above the threshold, based on linear extrapolation as done in the LLNA) were calculated as well as the IC50 for the concentration with 50% reduction in viability.

Table 2
Matricaria chamomilla extract optionally spiked with different spiking agents at different doses. Results from full dose-response analysis in the KeratinoSens™ assay.

Extract	Spiking component	Conc. of spiking component	I_{max}	EC 1.5 (ppm)[b]	Pos/neg	Reps. positive	IC50 (ppm)	EC 1.5 adjusted to spiked material
Chamomile	No		1.1	n.i.	0	0 of 9	>1000	
No extract[a]	Citral	100%	82.6	1.1	1	3 of 3	20.1	1.1
Chamomile	Citral	5%	108.4	26.5	1	3 of 3	836.1	1.3
Chamomile	Citral	2%	5.9	81.5	1	3 of 3	>1000	1.6
Chamomile	Citral	0.50%	1.9	225.3	1	3 of 3	>1000	1.1
No extract[a]	Cinnamic aldehyde	100%	15.0	6.4	1	3 of 3	40.3	6.4
Chamomile	Cinnamic aldehyde	5%	8.4	179.7	1	3 of 3	784.8	9.0
Chamomile	Cinnamic aldehyde	2%	2.9	516.1	1	3 of 3	>1000	10.3
Chamomile	Cinnamic aldehyde	0.5%	1.3	n.i.	0	0 of 3	>1000	>5
No extract[a]	Isoeugenol	100%	14.1	10.6	1	3 of 3	208.5	10.6
Chamomile	Isoeugenol	10%	4.6	99.3	1	3 of 3	789.2	9.9
Chamomile	Isoeugenol	5%	1.6	215.6	1	2 of 3	>1000	10.8
Chamomile	Isoeugenol	2%	1.4	n.i.	0	0 of 3	>1000	>20

[a] Indicates result for the pure spiking agent.
[b] n.i. indicates no induction above the 1.5-fold threshold up to the maximal dose tested (1000 ppm).